Uterine Contractile Activity In The Mare

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1998

Declaration

I hereby declare that the composition and experiments of this Thesis and the work presented in it are entirely my own with the exception of the prostaglandin assay that was carried out by Prof. H. Kindahl and the progesterone assay that was carried out by Ms S. Thomson. This Thesis has not been submitted for the purposes of obtaining any other degree or qualification from any other academic institution.

Elias Nikolakopoulos

Acknowledgements

Where does one start really? Firstly, I wish to thank my supervisor, Elaine Watson, for her relentless guidance over the past four years. With her essential and generous contributions to this thesis she has taught me a great deal. I would also like to thank Mo Jordan and Sheila Thomson for trying to making my life so much easier, at least most of the times. Thanks to the rest of the repro team, Dr. Lesley Anderson, Dr. Denise Lawler, Hanne Gervi-Pedersen, Sung-Eun Bae for their moral and practical support. From the Faculty, I wish to thank Prof. Halliwell and Dr. D. Cottrell for always being available and supportive and Mrs Kath Tracey, Lisa Brannan and Janet Hackel for being ever so helpful. A big thank you to the grooms, without whose help my life would have been much more difficult, and to the owners of the mares that let me use their mares for my experiments. I am grateful to all those beautiful animals that put up with me for so long....

I thank Prof. Hans Kindahl and his technician, Mrs Lotta Sundström, in Uppsala, Sweden, for processing my PGFM samples and Dr. Colin L. Gilbert et al. (Pauline, Margie, Tom and Tim), in The Babraham Institute, for accommodating me in a lab with the friendliest atmosphere, ever. It has been a real pleasure. Special thanks to Jane Goode for her patience and kindness in teaching me the RIA. Also special thanks to Orestis Papasouliotis for substantial help with statistics.

I owe unpayable gratitude to Ms. Sophia Lavranou, my lovely landlady, for having me as a son for all those years and for creating a home, so far away from home. My love and respect will always be with her. For their valuable presence in my home I thank Leda-Olympiada Vlachou, Rolando Carrera, Stamati Spirou, Dr. Borislav Georgiev, Ellie Katsoulis, Dr. Xulio Maside and Alexandra Stamou.

I have been lucky enough to be surrounded by a group of special people that have been there in good times, but most importantly in bad. A big thank you to my brothers and

sisters in arms, for being my teachers and friends: Dr. Parveen Sandhu, Dr. Demetrios Fragoyiannis, Dr. Spyridoula Athanasiadou, Alex Astaras, Alkistis Loukides, Dr. Sophie Gilbert, Lydia Michael, Demetrios Savvides, Dr. Christina Anagnostopoulou, Jane Lilian Rocha, Nick Diakakis, Nikos Nikolinakos, and Dr. Costas Papathanasiou amongst so many. Thank you all!

Special thanks to Alba Higgins and Punya Kaufeler for teaching me their art and making my life blossom in so many different ways.

The end of this thesis signifies the end of a long journey, my Odyssey, that started a bit more than 11 years ago. And what a journey it has been.... However, I would not have made it without the continuous support of some unique people. Last, but by no means least, I wish to thank my mother and father for their loving and unconditional support all those years. My great respect to my father, for depriving himself of a lot, so I could accomplish this very task. I dedicate this thesis to them and I hope I can return just a small part of their generosity.

In the loving memory

of those who fought,

but did not make it to this day...

ABSTRACT

UTERINE CONTRACTILE ACTIVITY IN THE MARE

Endometritis is the commonest reproductive disease in the mare resulting in decreased fertility and economic losses to the equine industry. Endometritis is manifested by accumulation of intrauterine fluid and cellular debris and its therapy is mainly aimed towards enhancing uterine clearance. Impaired uterine contractile activity (UCA) in susceptible mares has been shown to contribute to defective uterine clearance.

In this thesis the importance of UCA in uterine clearance was demonstrated by converting the uterus of a genitally normal mare, using clenbuterol, a β_2 sympathomimetic, into a susceptible uterus, after bacterial infusion. All clenbuterol-treated mares had intrauterine fluid collections 48 h after the infusion. Uterine contractile activity is mainly controlled by the action of the ecbolic hormones, oxytocin (OT) and prostaglandin F2α (PGF2α). The profiles of these two hormones were investigated in resistant and susceptible mares around artificial insemination (AI) and after OT injection. Other stimuli applied only to resistant mares included oestrous and dioestrous teasing, natural service, intrauterine saline infusion and manual manipulation of the genital tract. All stimuli caused OT release and there were no differences in OT profiles between resistant and susceptible mares. However, significantly fewer susceptible mares released PGF2α in response to endogenous OT release or exogenous OT administration.

Qualitative measurement of UCA has been reported in mares using ultrasonography and its effect on ecbolic hormone release and subsequently UCA was investigated. There was no evidence to show any effect of ultrasonography on UCA. Utilizing the same technique, differences in UCA before and after OT administration in oestrous resistant and susceptible mares were investigated. Oestrous resistant mares had higher baseline UCA and OT administration caused uterine spasm in all oestrous mares. However the

repeated use of OT at short time intervals caused uterine refractoriness. Daily OT administration in the early postovulatory period significantly affected UCA up to day 3 postovulation.

It was finally concluded that UCA is responsible for the clearance of uterine fluid from the uterus. Mares susceptible to endometritis have lower baseline uterine motility and UCA is restored slower after OT administration. There are no differences in the OT profiles between resistant and susceptible mares and different mechanical and psychogenic stimuli associated with reproductive events, seem to trigger OT release. However, most susceptible mares do not release PGF2α in response to endogenous OT release or exogenous OT. Oestrous resistant mares respond to OT administration with uterine spasm and PGF2α release. However in dioestrus, OT stimulates UCA only up to day 3 postovulation.

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Chapter 1

Literature Review

Physiology of the oestrous cycle in the mare (*Equus caballus*)

The oestrous cycle of the mare, as well as in other domestic species, is greatly controlled by levels of circulating hormones. Oestrogens, progesterone, FSH, LH and oxytocin are some of the most important regulators of reproductive function in the mare.

Mares are reproductively active for 6 months per year, from approximately April through October in the northern hemispere, although this varies considerably depending on the levels of light in every region. The oestrous cycle in the mare, lasts 22 days and consists of an oestrous and a dioestrous period, lasting approximately 7 and 15 days, respectively.

Ovulation and the formation of a corpus luteum (CL) indicates the end of the oestrous period and the beginning of dioestrus (Stabenfeldt *et al.*, 1972). It is accompanied by a sudden increase in progesterone levels and a steady decrease of circulating oestrogens (Plotka *et al.*, 1975). Circulating oestrogen concentrations rise for approximately 7 days, and reach maximum values 2 days before ovulation. LH levels reach a maximum 1 to 2 days after ovulation, and decrease over the next 4 to 6 days. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is produced by the endometrium and is responsible for the lysis of the corpus luteum that signalls the end of the dioestrous period (Douglas and Ginther, 1975). Progesterone, oestrogen and oxytocin are thought to be involved in the regulation and release patterns of $PGF_{2\alpha}$. Unlike in ruminants, and other species, there is no ovarian oxytocin in the mare (Stevenson *et al.*, 1991) and $PGF_{2\alpha}$ is delivered to the ovaries not through a local uteroovarian pathway but via the systemic route.

The shape of the equine uterus varies from Y to T shaped. The uterine body is located mainly in the pelvis, however it is partly deflected by the surrounding organs. Parity and age amongst many other parameters affect uterine reproductive function in the mare.

Endometritis is the commonest reproductive disease in the mare (Varadin, 1975) and its relation to uterine contractile activity will be in part examined in the present thesis.

Endometritis - an overview

Importance of endometritis

Equine endometritis has been described since the end of the last century by researchers in Germany (Merkt, 1957), Australia (Bain, 1945), in the UK and the United States (Dimmock, 1923; Dimmock, 1935; Jennings, 1941). In equine practice persistent endometritis is the commonest reproductive disease in the mare (Varadin, 1975) and has been ranked by practitioners as the third most common medical problem in horses after colic and viral respiratory disease (Traub-Dargatz *et al.*, 1991). A transient endometritis is an inevitable sequel to breeding (Ricketts and Mackintosh, 1987) that is manifested as an accumulation of intrauterine fluid, visible by transrectal ultrasonography. Significant amounts of intrauterine fluid have been shown to accumulate after mating in 14% of Thoroughbred mares (Zent, 1997) and in 43% of a mixed population of mares in the UK (Newcombe, 1997). Successful conception following breeding is dependent on the resolution of uterine inflammation by 144 to 156 hours postovulation in order to accept the descending embryo (Battut *et al.*, 1998). If the infection persists for more than five days after coitus it will prevent successful pregnancy by producing an embryotoxic environment (Adams *et al.*, 1987; McKinnon *et al.*, 1988b; Pycock and Newcombe, 1996b). Furthermore, premature release of prostaglandin (PG) F2α, as a result of a uterine inflammatory process (Neely *et al.*, 1979b), causes lysis of the corpus luteum necessary for the maintenance of pregnancy (Douglas and Ginther, 1975).

Endometritis and early embryonic death

Mares have the lowest fertility rate of all domestic species (Rossdale *et al.*, 1980) and despite the development of many types of new antibiotics and treatments, endometritis has remained a major cause of infertility in brood mares over the last 50 years (Dimmock, 1935; Bain, 1945; Merkt, 1957; Collins, 1964; Bain, 1966; Hughes and Loy, 1975). Subfertile or "repeat" breeding mares have a higher incidence of embryonic loss between days 15 and 50 after ovulation compared to other mares (Villahoz *et al.*, 1985). Genital infections are associated with early embryonic death, abortion and perinatal mortality as well as infertility (Knudsen, 1964b; Varadin, 1975; McKinnon *et al.*, 1994; Mattoras *et al.*, 1995). Different researchers have shown that embryonic loss and not fertilisation failure is the more important cause of infertility in mares, and that the embryonic loss is associated with uterine inflammation (Douglas, 1982; Squires *et al.*, 1982). In a study by Woods *et al.* (1985), subfertile and maiden mares were compared by uterine biopsy and embryo recovery attempts on Days 7, 8 and 9 after ovulation. Only the subfertile mares had indications of uterine pathology, and most of them had embryonic vesicles that were classified as abnormal. Decreased embryo viability rates have been reported for subfertile mares at Day 4 (Ball *et al.*, 1989). In another study the fertilisation rate (embryo recovery at Day 2) was the same for barren and normal mares but the estimated loss rate was higher for the barren group than the normal group (Ball *et al.*, 1986). Lower pregnancy and higher embryonic loss rates were observed in mares with uterine fluid collections at dioestrus, a classical sign of endometritis, as opposed to mares without such collections (Adams *et al.*, 1987).

Classification of mares

Until recently, mares were classified as resistant or susceptible to endometritis according to their ability to eliminate uterine infection within a certain period of time (Hughes and Loy, 1969; Peterson *et al.*, 1969). Mares that eliminated uterine infection, resulting in a

normal uterine environment were referred to as resistant, whereas mares failing to do this were referred to as susceptible to endometritis. However, the multifactorial nature of susceptibility to uterine infection has recently raised questions regarding accurate classification of "susceptible" and "resistant" mares (Troedsson *et al.*, 1995a). Susceptibility to persistent uterine infection can be suspected in mares with: 1) repeated uterine bacterial infections, 2) repeated failure to conceive after having been bred to a fertile stallion and 3) accumulation of fluid in the uterus, as detected by ultrasonography (Troedsson and Liu, 1995). The new model of classification divides susceptible mares into four separate categories based on clinical aspects of endometritis and according to the underlying pathophysiological mechanisms: 1) sexually transmitted diseases (STD), 2) chronic infectious endometritis, 3) persistent mating-induced endometritis (PMIE), and 4) chronic degenerative endometrosis (LeBlanc, 1997a; LeBlanc, 1997b; Troedsson 1998).

Contagious equine metritis is the best example of STD in mares and its treatment and control is not the subject of the present thesis. Chronic uterine infection occurs normally in older pluriparous mares where the uterine defence systems are not functioning properly and even normal genital flora can be the cause of chronic endometritis (LeBlanc, 1997a). Both STD and chronic infectious endometritis are diagnosed after the invading microorganism has started replicating in the uterine environment and can be treated with antibiotics. However, PMIE appears as a consequence of the mating process with impaired uterine clearance being the indicative symptom (Troedsson, 1997). Failure or neglect of treatment of PMIE mares may lead to chronic uterine infection or chronic degenerative endometrosis and negatively affect fertility (LeBlanc, 1997b).

Diagnosis of endometritis

The diagnosis of endometritis is a complex procedure that is based on selected parameters, including reproductive history, uterine cytology and bacteriology, physical examination and endometrial biopsy. As an additional method of assessing susceptibility to endometritis response to bacterial challenge has also been suggested (Troedsson and Liu, 1991).

Subfertile mares as diagnosed from their breeding history should be thoroughly examined. To correctly diagnose endometritis, a breeding soundness examination should be performed. That includes: 1) examination of the external genitalia of the mare, 2) transrectal palpation and ultrasound examination of the genital tract, 3) endometrial cytology, 4) endometrial bacteriology and 5) endometrial biopsy. In addition with the mare's history and breeding soundness examination results it is possible to predict the reproductive future of the mare (Kenney *et al.*, 1986).

Evaluation of the genital tract

The three valves of the mare's reproductive system (cervix, vaginovestibular sphincter, vulvar sphincter) represent the mechanical obstacles to the introduction of infection into the uterus. The failure of any of these three will result in the collapse of the entire uterine defence (Troedsson 1991). Poor vulvar conformation and thinning of the perineal body as well as conformational changes attributable to body condition may lead to urovagina (urine pooling in the vagina) or pneumovagina (aspiration of air and contaminants in the vestibule) (Troedsson and Liu, 1995). Reasons for failure of the vulvar sphincter are ageing (Hughes and Loy, 1969; Troedsson and Liu, 1995), multiple foaling, perineal lacerations, cervical stretching, or tearing. Any of those conditions will allow the contamination of the cranial vagina, which may result in the inflammation of the cervix and uterus, thus contributing to infertility. Other possible anatomical changes

such as an enlarged uterus suspended deeply over the brim of the pelvis in multiparous and old-aged mares may be another cause of the inability of the uterus to physically clear an infection (Rowson *et al.*, 1953; Medici *et al.*, 1991; Troedsson and Liu, 1991; Troedsson *et al.*, 1995b; LeBlanc *et al.*, 1998). Parturition and retention of the foetal membranes even for a few hours offer the bacteria the chance to migrate from the soil and the bedding into the uterus (Farrely and Mullaney, 1964; Williamson *et al.*, 1984).

Transrectal palpation and ultrasound examination of the genital tract

Detection of intraluminal fluid accumulation in mares by transrectal palpation was first reported by Knudsen (1964b). Transrectal ultrasonography allows the clinician to observe and evaluate in real time mode the integrity of the uterus and diagnose uterine pathology (McKinnon *et al.*, 1987) including the presence of uterine fluid accumulations (Ginther and Pierson, 1984). The presence of small amounts of fluid in the uterine lumen during oestrus, commonly observed by ultrasonography as a thin lining, has been suggested to resemble transudate (Reilas *et al.*, 1997) and it has been shown to have antibacterial activity (Strzemienski *et al.*, 1984). However, the presence of large amounts of uterine fluid detected with ultrasonography during oestrus and dioestrus has been demonstrated to reduce fertility in mares (Adams *et al.*, 1987; Pycock and Newcombe, 1996b; Newcombe, 1997). During oestrus, uterine fluid may suppress spermatozoal motility (Squires *et al.*, 1989) or may represent an environment which is ideal for the support of bacterial proliferation after breeding. When present during dioestrus, uterine fluid may cause premature luteolysis and decreased fertility (Adams *et al.*, 1987; Pycock and Newcombe, 1996b). In the postpartum mare, it may be associated with poor uterine involution and endometritis (McKinnon *et al.*, 1988b). McKinnon (1987) described a system of grading uterine fluid by quality from 1 to 4 according to its degree of ultrasonographic echogenicity which is correlated with the amount of debris or white blood cell infiltration into the fluid. Grade 1 fluid has large numbers of neutrophils and Grade 4 fluid has very few neutrophils (McKinnon *et al.*, 1987). It has

been shown that small amounts of grade 1 or 2 fluid decrease fertility (Squires *et al.*, 1989), while anechogenic (Grade 4) intrauterine fluid accumulations are commonly seen in oestrous mares and do not decrease conception rates (Reilas *et al.*, 1997).

Exfoliative endometrial cytology

Exfoliative endometrial cytology has proved to be a useful technique for the diagnosis of uterine inflammation. Several methods such as double-guarded swabbing, cotton tampons and low-volume saline uterine flush have been used to collect cells from the endometrial surface, each offering different advantages and disadvantages. Cytocentrifuge preparations or smears are prepared on a glass slide, and fixed with suitable cytology fixative. Normally the cellular populations found should be squamous and columnar epithelial cells and occasionally red blood cells. Active endometritis is indicated by the presence of inflammatory cells (Knudsen, 1964a; Knudsen, 1964b; Wingfield-Digby, 1978; Wingfield-Digby and Ricketts, 1982). Parameters used for the quantitative estimation of neutrophils in endometrial smears include percentage of neutrophils (Ricketts, 1981; Ball *et al.*, 1988), numbers of neutrophils per field (Knudsen, 1964a; Asbury, 1984; Brook, 1985) and ratio of neutrophils to epithelial cells (Wingfield-Digby, 1978; Asbury *et al.*, 1982; Couto and Hughes, 1984). Although cytology results are reliable and indicative of the presence of different cellular populations of the endometrium they should be evaluated together with the results of the uterine culture and endometrial biopsy.

Endometrial bacteriology

Culture of endometrial swabs represents another diagnostic tool for the diagnosis of endometritis. It appears that the bacterial flora of the uterus and cervix has changed very little in the past five decades (Hughes and Loy, 1969; Hughes and Loy, 1975; Ganjam *et*

al., 1980; Burns, 1983). Streptococcus zooepidemicus is the micro-organism most frequently isolated from mares with endometritis (Shin *et al.*, 1979; Wingfield-Digby and Ricketts, 1982). It is an extracellular opportunistic pathogen normally found on the external genitalia of clinically normal mares and stallions (Ricketts and Mackintosh, 1987). These organisms are periodically introduced into the uterus at coitus or in association with genital pathology (Ricketts and Mackintosh, 1987). Streptococci are highly adaptable host specific organisms that prefer selected anatomical sites (Garrioch, 1978). The primary host defences that limit streptococcal infections include non-specific mechanisms such as clearance of bacteria and specific antibody together with phagocytic cells that result in bacterial killing (Mims 1987). Bacteria have been commonly cultured from post-breeding samples (Bryans, 1962; Scott *et al.*, 1971; Newcombe, 1978; Ricketts, 1981; Wingfield-Digby and Ricketts, 1982) however, bacterial recovery remains low in resistant mares at subsequent specimen collections and bacterial contamination in the uterus is minimal 6 hours after breeding (Kotilainen *et al.*, 1994). In susceptible mares large numbers of bacteria are recovered at 48 and 96 hours after inoculation or breeding (Hughes and Loy, 1969; Evans *et al.*, 1987; Williamson *et al.*, 1987). Infection in the reproductive tract of the mare is due to various factors. In many species, including the mare, breeding, either naturally or via artificial insemination, provokes a strong inflammatory response (Cohen 1984; Brook, 1985; Nikolakopoulos and Watson, 1997b) probably induced by antigenic material in the semen (Troedsson *et al.*, 1995b; Troedsson *et al.*, 1995d). Although both bacteria and spermatozoa stimulate neutrophils chemotaxis into the uterus (Blue *et al.*, 1984; Pycock and Allen, 1990; Troedsson *et al.*, 1993c; Troedsson *et al.*, 1995d), highest neutrophil numbers were obtained when the uterus was infused with frozen semen.

Endometrial biopsy

Endometrial biopsy remains the definitive method for assessing inflammatory and chronic degenerative endometrial disease (Kenney and Ganjam, 1975; Gordon and

Sartin, 1978; Kenney, 1978; Ricketts *et al.*, 1978; De la Concha-Bermejillo and Kennedy, 1982; Kenney *et al.*, 1986; VanCamp, 1988). As mares age, chronic irreversible changes occur within the endometrium (Ricketts, 1975; Blanchard *et al.*, 1987; Williamson, 1989; Dybdal *et al.*, 1991; Held and Rohrbach, 1991). Such changes are of the following three types: endometrial fibrosis, lymphatic lacunae, and cystic dilatation of glands (Watson, 1994). Lesions are classed as normal if they fall within acceptable limits but depending on their severity, they may significantly decrease the ability of a mare to carry a foal to term (Kenney, 1978; Kenney *et al.*, 1986). Neutrophils are the predominant cell type in the uterine lumen in an acute inflammation. In a chronic reaction types, numbers and location of cells are probably determined by the nature, distribution, and persistence of the stimulant with lymphocytes being the predominant cell type (Kenney, 1978). Recently, a new classification method has been suggested based on the pathogenesis of endometritis and involving the subsequent fertility potential of the mare (Ricketts and Barrelet, 1995).

Last but not least, endometritis can result as an iatrogenic infection. Veterinary manipulations like gynaecological examination and artificial insemination may cause infection (Bruner, 1951; Collins, 1964; Farrely and Mullaney, 1964; Bain, 1966; Hughes and Loy, 1975; Williamson *et al.*, 1984; Williamson, 1989) by introducing pathogens in the reproductive tract. Other characteristics that predispose mares to repeated uterine infections include long oestrus, with a relaxed cervix that is a relatively weak barrier against bacterial invasion during oestrus compared with that of other domestic species (Knudsen, 1964b; Troedsson *et al.*, 1995b). Different aspects of the disease's aetiology, pathology and treatment have been investigated (Bruner, 1951; Rowson *et al.*, 1953; Merkt, 1957; Farrely and Mullaney, 1964; Hughes and Loy, 1975; Troedsson and Liu, 1995; Troedsson, 1997). Although a lot of progress has been made in understanding the complex mechanisms behind its pathogenesis, equine endometritis remains a significant reproductive disease resulting in reduced fertility (Colbern *et al.*, 1987; Adams *et al.*, 1987; Pycock and Newcombe, 1996b).

Uterine Defence Mechanisms

Uterine defence mechanisms against infections have been investigated and described in the mare: cellular mechanisms (Asbury *et al.*, 1984; Smith, 1984; Cheung *et al.*, 1985; Liu *et al.*, 1986; Watson, 1988b), antibody-mediated mechanisms (Asbury *et al.*, 1980; Watson *et al.*, 1987a; Williamson, 1989), as well as physical clearance (Evans *et al.*, 1987; LeBlanc *et al.*, 1989). Several aspects of the neutrophil function have been investigated and compared between mares susceptible and resistant to chronic uterine infection. They include chemotactic and phagocytic properties of peripheral and uterine derived neutrophils (Troedsson and Liu, 1991).

Cellular and antibody-mediated defence mechanisms

As with inflammatory processes in other organs, an important first line of defence within the mare's uterus appears to be ingestion and killing of bacteria by neutrophils (Hughes and Loy, 1969; Peterson *et al.*, 1969). Neutrophil phagocytosis includes mobilisation, and attraction of neutrophils, recognition and opsonization of the bacteria and finally ingestion, killing and subsequent removal from the uterus (Asbury *et al.*, 1982; Asbury *et al.*, 1984; Strzemienski *et al.*, 1984; Cheung *et al.*, 1985; Liu *et al.*, 1985; Liu *et al.*, 1986; Asbury and Hansen, 1987; Watson *et al.*, 1987a). Neutrophils can be seen marginating in capillaries in endometrial biopsy samples obtained during oestrus, and they migrate rapidly into the uterine lumen in response to challenge (Kenney, 1978; Katila, 1994). Chemotactic and phagocytic functions of neutrophils have been suggested to be functionally impaired in mares potentially susceptible to chronic uterine infections. The functional deficiencies described are reduced migration capabilities as determined by the ability of uterine derived neutrophils to migrate through chemotactic chambers (Liu *et al.*, 1985; Liu *et al.*, 1986) and the reduced ability to phagocytose as determined by chemiluminescence (Asbury *et al.*, 1982) and yeast engulfment studies (Smith, 1984; Cheung *et al.*, 1985). Also in the study by Cheung et al. (1985) it has been suggested that both the deformability of the cells and their ability to ingest and kill

organisms may be changed when the cells migrate through the endometrium of mares with degenerative uterine changes. By contrast, results from a study by Troedsson (1983), clearly demonstrated that uterine neutrophils from susceptible mares are fully functional, given the right environment. In fact, uterine neutrophils from susceptible mares performed better than neutrophils from resistant mares, with regard both to both phagocytosis and chemotaxis. The phagocytic capacity of uterine neutrophils collected 4 hours after an infection was greater for susceptible than resistant mares. Also a study by Asbury and Hansen (1987) failed to show a difference in the phagocytic activity between uterine neutrophils from susceptible and resistant mares. Opsonization is the process by which a number of organisms are bound together and to the membrane of the neutrophils. It has been suggested earlier that inefficient opsonization is a factor in susceptibility to endometritis (Asbury *et al.*, 1984) since an impaired opsonizing capacity of uterine secretions has been observed in the susceptible mares (Watson *et al.*, 1987a; Troedsson *et al.*, 1993c). The susceptible mare is deficient in the content of opsonins to a degree which allows bacteria to become established and produce endometritis (Asbury *et al.*, 1982). Impaired chemotactic properties were suggested by *in vitro* studies in susceptible mares (Liu *et al.*, 1985). The mechanisms of chemotaxis and opsonization have been further investigated by other researchers (Lees *et al.*, 1986; Watson, 1987a; Watson, 1987c; Watson *et al.*, 1987b; Watson, 1988a; Kao 1989; Troedsson *et al.*, 1990).

Uterine clearance and uterine contractile activity (UCA)

The importance of uterine clearance was first suggested by Irvine *et al.* (1981) and Watson (1987b) who suggested that susceptible mares have impaired UCA. Uterine contractile activity is responsible for the mechanical drainage of cellular debris and uterine fluid from the uterine lumen as well as movement and proper placement of sperm, ova, and conceptus and, ultimately, expulsion of the foetus. It enhances uterine clearance, facilitating drainage either by mechanical expulsion through the open cervix

(Evans *et al.*, 1987; Troedsson and Liu, 1991) or by compressing the lymph vessels, which move material centrally to the lymph nodes (Guyton 1991). Delayed physical clearance of intrauterine fluids and bacteria through the cervix is implicated as a cause of recurrent endometritis (Evans *et al.*, 1987; Allen and Pycock, 1988; Troedsson and Liu, 1991; LeBlanc *et al.*, 1994a) and may be myometrial in origin (Troedsson and Liu, 1991). The intensity and synchrony of UCA in response to infection might be impaired in susceptible mares. The prolonged accumulation of destructive inflammatory products could negatively affect the defence mechanisms of the uterus, including an increased catabolism of complement components and immunoglobulins as well as impaired neutrophil function (Troedsson *et al.*, 1992). Studies of young maiden and older multiparous mares demonstrate a significant delay in mechanical clearance of non-antigenic markers from the uteri of the aged, multiparous mares (Evans *et al.*, 1987). The results suggested that the physical ability of the uterus to completely eliminate bacteria is age-related (Evans *et al.*, 1987; LeBlanc *et al.*, 1989; LeBlanc *et al.*, 1994a), but they do not demonstrate the differences that may occur between mares susceptible and resistant to chronic uterine infection (Troedsson and Liu, 1991). Using non-antigenic microspheres LeBlanc *et al.* (1989) suggested that no differences in uterine clearance exist between mares potentially resistant and susceptible to chronic uterine infection. By contrast a similar study by Troedsson and Liu (1991) demonstrated a significant decrease in clearance by susceptible mares. The fact that the former study (LeBlanc *et al.*, 1989) was performed during post ovulation period, while Troedsson's in oestrus, may be indicative of the different clearance properties of the resistant and susceptible uterus at different stages of the cycle. Also it was demonstrated that in dioestrus lymphatic drainage is decreased in susceptible mares (LeBlanc *et al.*, 1995c). These findings agree with a study in mares which showed that cervical drainage was enhanced by oestrogen stimulated relaxation of the cervix during oestrus and inhibited by progesterone stimulated closure during dioestrus (Winter, 1982). Impaired uterine clearance of mares susceptible to PMIE represents a model of study that might help us understand better the underlying pathophysiologic mechanisms of endometritis.

Structure of the myometrium

The myometrium of most species including the horse consists of two distinct smooth muscle layers with a vascular zone in between: an outer longitudinal layer and an inner circular layer whose muscle fibres are arranged respectively, parallel to or concentrically around the long axis of the uterus (Finn *et al.*, 1975). The two muscle layers are composed of smooth muscle cells embedded in a connective tissue matrix and arranged into muscle bundles that are interconnected: those of the longitudinal myometrium interlace to form a network while those of the circular myometrium appear less clearly arranged (Garfield *et al.*, 1985). The physiology, innervation and contraction patterns of the two muscle layers is quite different as has been demonstrated in the rat (Osa and Katase, 1975; Anderson *et al.*, 1981; Bengtsson, 1982; Izumi, 1985; Crankshaw, 1987; Tuross *et al.*, 1987), guinea-pig (Hall and Pennefather, 1990), rabbit (Nesheim, 1974) sow (Taneike *et al.*, 1991). In the mare, an *in vitro* mechanical testing procedure for the investigation of spontaneous contractile activity of the two myometrial layers indicated that both muscle layers contract independently of one another and appear to be modulated by endocrine factors (Liu *et al.*, 1998). The individual uterine smooth muscle cells are the physiological units of contractile function (Marshall and Csapo, 1961; Csapo, 1962). Their number and size changes during the oestrous cycle and pregnancy. The physiology of muscle contraction (Csapo, 1962) and especially of uterine smooth muscle cells (Csapo and Gergely, 1950; Marshall and Csapo, 1961) has previously been described and investigated. The sequence of contraction and relaxation of the myometrium results from the cyclic depolarisation and repolarisation of the membranes of the muscle cells. The spontaneous electrical discharges in uterine muscle consist of intermittent bursts of spike-action potentials (Marshall and Csapo, 1961; Kuriyama and Suzuki, 1976).

Methods of measuring UCA

Measurement of UCA was demonstrated initially using an intra-cavital fluid-filled bag by Schatz (1872) in the human pregnant uterus. Since then different external non-invasive and internal invasive methods have been used to record parameters of contractions of the human uterus and to quantitate UCA.

Electromyography measures the electrical activity of the myometrium and has been used for the quantitation of UCA in several species including the ewe (Naaktgeboren *et al.*, 1973; Lehrer *et al.*, 1978; Garcia-Villar *et al.*, 1984; Sigger *et al.*, 1984; Faltsi and Brikas, 1990; Gilbert *et al.*, 1992), rat (Kuriyama and Suzuki, 1976) and sow (Taverne *et al.*, 1979a; Claus *et al.*, 1989). In the mare, electromyography has been used to study uterine contractions in nonpregnant or ovariectomized mares (Taverne *et al.*, 1979b; Toutain *et al.*, 1983; Haluska *et al.*, 1987; Jones *et al.*, 1991; Troedsson *et al.*, 1993a; Troedsson *et al.*, 1993b; Troedsson *et al.*, 1994).

Intrauterine pressure measurements have been extensively used to study UCA in women (Cibils, 1967; Crawford, 1975; Seitchik and Chatkoff, 1976; Barclay *et al.*, 1977; Smith, 1984; Bulletti *et al.*, 1997) as well as in the sow (Zerobin and Spoerri, 1972) and the monkey (Hsu *et al.*, 1989). In the mare intrauterine pressure has been used in recent years to describe and quantitate UCA (Goddard and Allen, 1985; Goddard *et al.*, 1985; Goddard and Allen, 1988; Ley *et al.*, 1988; Ko *et al.*, 1989; Paccamonti *et al.*, 1997). Toutain *et al.* (1983) demonstrated a close relationship between electrical and mechanical activity in uterine smooth muscle. Jones *et al.* (1991) as well as Taverne *et al.* (1979b) and Troedsson *et al.* (1993a) found that uterine activity changes with the stage of the oestrous cycle and differs between different parts of the uterus. Goddard *et al.* (1985) and Ko *et al.* (1989), using pressure balloons and pressure transducers, found no difference in the uterine activity during the oestrous cycle measuring intrauterine pressure. Circumferential gauge devices have also been used for UCA quantification in the mare, however their use is limited (Capraro *et al.*, 1977).

Different units for UCA measurement have been reported in the literature based on data collected with one of the above mentioned methods. However, a universal unit has not yet been accepted although attempts were made to establish the Montevideo Unit (Caldeyro-Barcia *et al.*, 1957), the Alexandria Unit (El-Sahwi *et al.*, 1967), the area under the uterine pressure curve (Hon and Paul, 1973) and the mean active pressure (Phillips and Calder, 1987).

All methods express myometrial contractions as changes in either electrical activity, intrauterine pressure or the circumference of the uterine horns. These approaches are limited by their need for surgical intervention, with external impingement upon the reproductive tract or by invasion of the uterine lumen and their disadvantages have been reported (Csapo, 1970; Wolfs *et al.*, 1971; Zerobin and Spoerri, 1972; Devedeux *et al.*, 1993).

Ultrasonography

The introduction of ultrasonography in equine reproduction increased the veterinary diagnostic potential. Real-time images collected by ultrasonography of the uterus and ovaries allows earlier pregnancy diagnosis and more accurate information about the state of the genital tract (McKinnon *et al.*, 1987; McKinnon *et al.*, 1988a; McKinnon *et al.*, 1988b).

Ultrasonography has also been used for the quantitatation of UCA. In women, transvaginal and transabdominal ultrasonography have proved to be reliable methods for the evaluation of UCA (Birnholz, 1984; Abramowicz, 1990; Lyons *et al.*, 1991; Salamanca and Beltran, 1995; Kunz *et al.*, 1996; Leyendecker *et al.*, 1996; Kunz and Leyendecker, 1997) and in the mare it has successfully been used to study uterine contractions during the oestrous cycle and early pregnancy (Cross and Ginther, 1987;

Cross and Ginther, 1988; Griffin and Ginther, 1990; Gastal *et al.*, 1998). In mares, UCA was measured and scored from 1-minimal to 5-maximal, using a video recording device. Uterine contractile activity was recorded for 1 minute and then graded by different researchers (Cross and Ginther, 1988; Griffin and Ginther, 1990). The high repeatability of results between the independent operators indicates that ultrasonography is a valid approach to the quantitation of UCA in mares (Griffin and Ginther, 1990). In an ultrasonic study by Cross and Ginther (1988), the period of maximal uterine activity during the oestrous cycle of pony mares (Days 14 to 18) corresponded temporally with the expected period of luteolysis, the time when $PGF_{2\alpha}$ is released into the uterine vein (Douglas and Ginther, 1976) and uterine lumen (Berglund *et al.*, 1982). This finding concurs with the electromyographic finding that myoelectrical activity was at its greatest during luteolysis, with high frequency phases of electrical discharges (Taverne *et al.*, 1979b). In early pregnant pony mares, the period of maximal ultrasonically detectable uterine activity (Days 10 to 14) occurred earlier than in nonpregnant mares (Cross and Ginther, 1988) and coincided with the reported period of maximal embryo motility (Leith and Ginther, 1985).

Control of UCA

The electrical activity of any smooth muscle tissue may be modulated by either myogenic, neurogenic or hormonal mechanisms. Myogenic activity refers to spontaneous activity which occurs in the absence of any neural or hormonal input, like basic intrinsic excitability, or the ability to produce spontaneous and rhythmic contractions. The signal for myogenic contractions originates in the spontaneous oscillation of the membrane potential which is provided by pacemaker cells. Neurogenic and hormonal activity are attributed to the neural or humoral control systems which are superimposed on the myogenic mechanisms to modify myometrial activity (Garfield *et al.*, 1988; Nesheim, 1974; Taneike *et al.*, 1991). An extensive system of adrenergic nerves, that the uterus is supplied with, is responsible for the

neurogenic control of the uterine contraction (Chow and Martin, 1981). Physiologically, the stimulation or inhibition of myometrial contractility occurs through the influence or manipulation of all these three mechanisms (Garfield *et al.*, 1988). The ecbolic hormones, prostaglandin $PGF_{2\alpha}$ and oxytocin, have tremendous influence on uterine defence mechanisms through their effect on myogenic properties and control of the myometrium (Csapo and Corner, 1953; Csapo, 1956b; Marshall and Csapo, 1961; Carsten and Miller, 1981; Tuross *et al.*, 1987). The effects of these hormones on uterine contractility are considered separately below.

Effect of ovarian steroids: Progesterone and Oestrogen

Progesterone is a primary secretory product of the corpus luteum. The effect of progesterone treatment on uterine muscle is known to consist of a suspension of the ability to propagate action potentials and also an induction of asynchronous activity (Marshall, 1959; Csapo 1961). Progesterone increases electrical resistance between the myometrial cells (Ichikawa and Bortoff, 1970) and predisposes the uterus to infection by constricting the cervix and inhibiting uterine contractions (Evans *et al.*, 1987). Progesterone has been shown to have a quieting effect on uterine contractions in most species (Rodriguez-Martinez *et al.*, 1987) and therefore a deleterious effect on uterine defence mechanisms. Initial reports on cattle (Rowson *et al.*, 1953) indicated that pyometra would develop after intrauterine inoculation of a pyogenic organism in dioestrous females but not on oestrous females. However, in mares, an electromyographic study (Taverne *et al.*, 1979a) demonstrated definite phases of electrical activity throughout dioestrous. Inhibition of uterine defence mechanisms by progesterone was related to a decrease in a noncellular bactericidal factors, slower neutrophil chemotaxis to uterine tissues and a decrease in uterine inflammatory response (Ganjam *et al.*, 1980; Watson *et al.*, 1987a; Watson *et al.*, 1987c; Watson *et al.*, 1988a; Watson *et al.*, 1988b).

Oestrogen stimulates uterine contractions during the oestrous cycle (Cross and Ginther, 1987) and also stimulates the local immune system (Evans *et al.*, 1986). Increased myometrial activity reportedly occurs during oestrous in many species (Rodriguez-Martinez *et al.*, 1987; Anderson, 1978) including the mare (Nikolakopoulos and Watson, 1998). Taverne *et al.* (1979b) did not find any obvious relationship between circulating oestrogen concentrations and the electromyographic changes registered in the cycling mare. An indirect regulation of myometrial activity by oestrogen and progesterone, through their effect on oxytocin and prostaglandins, is well documented in women and rats (Fuchs, 1987; Maggi *et al.*, 1991).

Effect of oxytocin (OT) and prostaglandin $F2\alpha$ ($PGF2\alpha$) on UCA

Oxytocin (OT) is a nine amino acid peptide, Cys-Tyr-Phe-Glu(NH_2)-Cys-Pro-Lys-Gly(NH_2). Oxytocin is primarily synthesized in the paraventricular (PVN) and supraoptic (SON) hypothalamic nuclei where it is transcribed as part of a precursor molecule, neurophysin (Gainer *et al.*, 1994). Magnocellular OT neurons are located in the PVN and SON projecting and releasing OT from their processes (Buijs *et al.*, 1983) to a broad array of central targets such as the olfactory bulb, the telencephalon the brainstem and the spinal cord (Buijs and Van Heerikhuize, 1982; Swanson and Kuypers, 1980) and from the posterior pituitary into the general circulation (Bargmann and Scharrer, 1951). Although all placental mammals secrete OT from the posterior pituitary gland, the relative importance of the uterus and ovary as sources of OT may differ between species.

Ovarian oxytocic activity was first described by Ott and Scott (1911), and was not re-examined until the early eighties when two different groups found out that acidic extracts of bovine (Fields *et al.*, 1980) and ovine (Wathes and Swann, 1982) CL stimulated uterine contractions. Its presence was verified in the subsequent years (reviewed by Wathes, 1989) by using different methods in the cow (Fields *et al.*, 1983),

ewe (Sheldrick and Flint, 1983; Flint and Sheldrick, 1983), goat (Homeida, 1986), primates (Shukovski, 1992) and sow (Pitzel and et al. 1984). Oxytocin in the ruminant ovary is located and confined to the large luteal cells and in the pre-ovulatory follicle and OT staining was present exclusively in the granulosa (Wathes *et al.*, 1986) as opposed to the mare where no oxytocin was found in the preovulatory follicle (Stock *et al.*, 1995). A study by Stevenson et al. (1991) confirmed that the ovary of the mare is not a source of OT. However, recent studies have identified (OT) mRNA in the uterus of sows (Boulton *et al.*, 1996), women (Chibbar *et al.*, 1993), and rat (Lefebvre *et al.*, 1992). Furthermore, evidence that OT is also synthesised outwith the hypothalamo-neurohypophysial axis has been reported in the mare (Behrendt *et al.*, 1997). Immunostaining for neurophysin, the OT precursor molecule, suggested that OT is synthesised only by the luminal epithelium and the epithelium of superficial glands of the oestrous mare and not the myometrium (Watson *et al.*, 1998).

Uterine OT receptors

The location of the OT binding sites within uterine tissues may differ with species, however they appear to be located on the plasma membrane of the smooth muscle cells (Lehrer *et al.*, 1978). In the rat (Soloff and Swartz, 1974; Soloff, 1977) and the sow (Soloff and Swartz, 1974), binding sites were found only in the myometrium. In the mare (Stull and Evans, 1986), as in the ewe (Roberts *et al.*, 1976) and the woman (Fuchs, 1985), uterine oxytocin receptors (OTr) are present both in the endometrium and the myometrium.

The ovarian steroids oestrogen and progesterone control OTr concentration/density and their sensitivity/affinity to OT in the uterus in most species, although there are considerable species variations. Endometrial OT binding is maximal at the time of oestrus and lowest at the time of luteolysis in cows (Fuchs *et al.*, 1990; Jenner *et al.*, 1991), sheep (Roberts *et al.*, 1976; Sheldrick and Flint, 1985) and the rabbit (Small *et*

al., 1978). However, in the mare, as in women (Fuchs and Behrens, 1993), endometrial OTr concentrations vary during the oestrous cycle. In the mare endometrial OTr concentrations are highest between 14 and 17 days after ovulation (Stull and Evans, 1986; Sharp *et al.*, 1994) and remain significantly lower from ovulation to day 12, being lowest in oestrus (Sharp *et al.*, 1994). In the same study the affinity of endometrial OTr was reported not to vary during the oestrous cycle (Sharp *et al.*, 1994).

Release and function of oxytocin

The pulsatile secretion of OT from the posterior pituitary and the ovaries has been described in ruminants (Mitchell *et al.*, 1982; Walters *et al.*, 1984; Wathes 1989). There is little agreement on the amplitude and frequency of episodes of OT release in the mare or on concentrations at different stages of the cycle (Burns *et al.*, 1981; Tetzke *et al.*, 1987; Stevenson *et al.*, 1991; Alexander *et al.*, 1995; Sharp *et al.*, 1997). Concentrations of OT in cycling mares have variously been reported to be highest at oestrus (Burns *et al.*, 1981) or dioestrus (Tetzke *et al.*, 1987), or to remain at low levels throughout the cycle (Stevenson *et al.*, 1991). Differences in these reports might be due to the OT extraction and assaying procedures used or to blood sample regimens. Plasma OT concentrations collected from the intercavernous sinus were 8 fold higher than jugular blood plasma samples (Vanderwall *et al.*, 1998).

Oxytocin has long been associated with uterine contractions during parturition and milk ejection during nursing. Oxytocin is released at the expulsive stage of labor (Haluska and Currie, 1988) and just prior to the rupture of placental membranes (Haluska, 1989). Exogenous OT administration is used to promote fetal expulsion by enhancing UCA (Pashen, 1982). In the mare, suckling elicited an increase in the intrammammary pressure and was correlated with OT release (Ellendorff and Schams, 1988). Oxytocin binds to uterine OTr that are located in the endometrium and myometrium of the mare (Stull and Evans, 1986; Sharp *et al.*, 1994) and provokes uterine contractions by

increasing the influx of calcium into the myometrial cells (Csapo, 1962). Oxytocin also stimulates UCA indirectly by mobilising arachidonic acid and initiating the production of uterine $PGF2\alpha$ (Sharma and Fitzpatrick, 1974; Roberts *et al.*, 1976). Furthermore OT release was recently associated with the onset of maternal behavior in sheep (Keverne *et al.*, 1982) and rats (Pedersen *et al.*, 1982), and it has been shown that it can facilitate different types of behavior (Witt and Insel, 1990; Argiolas and Gessa, 1991; Insel, 1992; de Wied *et al.*, 1993; Leckman *et al.*, 1994; Landgraf, 1995), including reproductive behaviour (Argiolas *et al.*, 1988; Witt and Insel, 1991; Carter, 1992; Caldwell *et al.*, 1994a; Caldwell *et al.*, 1994b; Nishimori *et al.*, 1996; Insel *et al.*, 1997).

- *Prostaglandin $F2\alpha$ ($PGF2\alpha$)*

Regulation of $PGF2\alpha$ secretion is different between ruminants and mares due to the lack of OT from the ovary of the mare (Stevenson *et al.*, 1991). Oxytocin elicits $PGF2\alpha$ release from the equine endometrium *in vitro* (King and Evans, 1987; Franklin *et al.*, 1989) and *in vivo* at different stages of the cycle (Betteridge *et al.*, 1985; Goff *et al.*, 1987; Goff *et al.*, 1993; Nikolakopoulos *et al.*, 1998b) mediated by the OTr (Sharp *et al.*, 1994; Sharp *et al.*, 1997; Starbuck *et al.*, 1998). Centrally released OT initiates the arachidonic acid cascade, through the OTr, that will lead to the production and release of $PGF2\alpha$. Different mechanical stimuli such as embryo transfer (Betteridge *et al.*, 1985; Kask *et al.*, 1995; Kask *et al.*, 1997), endometrial biopsy (Sharp *et al.*, 1994) and intrauterine manipulations (Neely *et al.*, 1979b; Pascoe *et al.*, 1989) have been shown to elicit $PGF2\alpha$ release, as measured by its main initial plasma metabolite 15-keto-13,14-dihydro-$PGF2\alpha$ (PGFM) (Granstrom and Kindahl, 1982). A temporal relationship between OT and $PGF2\alpha$ release has been demonstrated in the mare (Sharp *et al.*, 1994; Sharp *et al.*, 1997; Vanderwall *et al.*, 1998) however definite conclusions have not been drawn yet.

Both $PGF2\alpha$ and OT have been implicated as ecbolic agents in mares (Troedsson *et al.*, 1995e). Prostaglandins are known stimulants of myometrial activity in the cow (Rodriguez-Martinez *et al.*, 1987) and in the mare (Capraro *et al.*, 1977). Myometrial electrical activity (Taverne *et al.*, 1979b) and intrauterine pressure (Goddard *et al.*, 1985) in mares were increased following injection of $PGF2\alpha$. Furthermore, uterine contractions in nonpregnant mares increased in late dioestrus (Cross and Ginther, 1988) at approximately the time prostaglandins are released into the uterine vein (Douglas and Ginther, 1976).

Objectives

The objectives of the research that will be described in the following chapters are:

1) to determine the importance of UCA in the clearance of experimentally-induced uterine infection in genitally normal mares and mares with reduced UCA

2) to determine the release patterns of the ecbolic hormones OT and $PGF_{2\alpha}$ at mating, teasing and other reproductive events in the oestrous mare

3) to determine the release patterns of the ecbolic hormones OT and $PGF_{2\alpha}$ at AI in mares resistant and susceptible to persistent mating-induced endometritis

4) to compare uterine responses of resistant mares to AI and natural service with that of susceptible mares to AI

5) to determine the validity of ultrasonography as a technique for evaluating UCA in the mare and then to monitor ultrasonographically the effect of OT administration on UCA in oestrous and dioestrous mares

6) to investigate the effect of OT on UCA in the early postovulatory period in the mare.

Chapter 2

Materials and Methods

Semen collection

Semen was collected from a fertile Thoroughbred-cross stallion. The same stallion was used for all inseminations. On the day of collection, the gel was separated by filtration and the semen was then diluted with Kenney's skim milk semen extender (containing 1 mg ticarcillin per ml) to a concentration of 25 million progressively motile sperm per ml in a total volume of 40 ml. The extended semen was chilled overnight in an insulated container (Equitainer, Hamilton-Thorn, Beverley, MA, USA). Cultural examination of raw semen demonstrated mixed bacterial growth. After 48 hours no growth was demonstrated either in extended semen incubated overnight at 37°C or extended semen chilled overnight.

Uterine Flush

The uterus was flushed with 60 ml PBS via a catheter after preparation of the genital area and the same amount of fluid was recovered into a sterile bottle. The volume of the recovered fluid was measured and the degree of cloudiness was graded visually on a scale from 0 (crystal clear) to 5 (purulent). Two well mixed 15 ml aliquots of the recovered uterine flush were processed further. The first aliquot was centrifuged at 200 *g* for 15 min, after which the supernatant was carefully removed and the cell pellet was resuspended in 1 ml sterile PBS. Total cell numbers were counted using a haemocytometer. Cytospin preparations were made and stained with Diff Quick (Franklin Medical, High Wycombe, UK) and the percentage of neutrophils to epithelial cells was determined. The second aliquot was centrifuged at 2000 *g* for 15 minutes and resuspended in 0.5 ml sterile PBS. The sample was then vortexed and streaked onto blood agar plates. The plates were then incubated aerobically at 37°C and checked for bacterial growth 24 and 48 hours later.

Sample Handling

Blood samples were collected into evacuated heparinised tubes and immediately placed on ice until separation. The samples were centrifuged at 2000 *g* for 15 minutes at 4°C. An aliquot of plasma was acidified with 10M acetic acid (10 μl ml $^{-1}$ plasma) for OT assay. Mild acidification of blood plasma has been shown to improve OT recovery rates in the ewe (de Winter *et al.*, 1995) and goat (Homeida and Cooke, 1984). All samples were frozen at -70°C and subsequently stored at -20°C until assayed.

OT Assay

Oxytocin was extracted from plasma using C_{18} SepPak cartridges (Waters Chromatography, Milford, MA, USA). The samples were thawed and centrifuged before being extracted in order to remove plasma debris. Oxytocin was extracted from 2 ml plasma samples using a solution of 80% methanol and 4% acetic acid. The samples were then dried in a speed-vacum evaporator and stored in dessicated environment. The samples were reconstituted at a later date and the radioimmunoassay was carried out as described by Thornton *et al.* (1986) using an antiserum described previously (Sheldrick and Flint, 1981). The extraction recovery rate was 74.8%. The detection limit for the assay was 0.8 pg/ml. The intra- and inter-assay coefficients of variation were 6.6 and 11.7 %, respectively.

PGFM Assay

Synthesis and release of $PGF_{2\alpha}$ can be monitored by analysing metabolites in peripheral (jugular) plasma in the horse (Neely *et al.*, 1979; Goff *et al.*, 1984). The main initial

metabolite of $PGF2\alpha$ is 15-ketodihydro-$PGF2\alpha$ and this metabolite was analysed in unextracted plasma by radioimmunoassay (Kindahl *et al.*, 1976; Granström & Kindahl, 1982). The antiserum cross- reacted 16% against 15-keto-$PGF2\alpha$, 4% against 13,14-dihydro-$PGF2\alpha$, 0.4% against $PGF2\alpha$, and 1.7% with 15-ketodihydro-PGE_2, a major metabolite of PGE_2. Other prostaglandins tested cross-reacted less than 0.1%. The detection limit of the assay was around 60 pmol/l equivalent to 20 pg/ml for analyses of 0.2 ml plasma. The intra- and inter-assay coefficient of variation were 6.6 and 11.7 %, respectively.

The equine plasma was analysed in 0.2 ml aliquots or in dilutions made in Tris-HCl buffer. To facilitate for the precipitation of the bound fraction when using polyethylene glycol for separation, all dilutions were made in the buffer containing 0.25% bovine gamma globulin. To avoid interference by heat-labile compounds in horse plasma and to increase parallelism in the assay, all samples were heat-treated for 30 min at 45°C (Neely *et al.*, 1979). This procedure had no effect on the standards.

Progesterone Assay

Progesterone concentrations were measured by radioimmunoassay directly in plasma without extraction using a technique previously described (Corrie *et al.*, 1981) and modified by Law et al. (1992). Anti-progesterone antiserum was kindly provided by the Scottish Antibody Production Unit, Carluke. The main cross-reactivities of the progesterone antiserum were with 5-pregnan-3,20-dione (9.5%), 11-deoxycorticosterone (6.2%) and 17-hydroxyprogesterone (3.4%). Progesterone standards were prepared in ovariectomised mare plasma. The sensitivity of the assay was 0.5 ng/ml and the intra- and inter-assay coefficients of variation were 9 and 12.6%, respectively.

Videotape analysis

Uterine contractile activity was assessed and scored from 1 minute segments of videotape. Uterine contractile activity can be detected by transrectal ultrasonography when the uterine body is viewed longitudinally. The changes in uterine echotexture during the cycle significantly alter the ultrasonographic image of the uterus. In oestrus, the greatest part of the image is occupied by the oedematous endometrial folds on both sides of the uterine lumen, while the myometrium cannot be clearly defined. By contrast, in dioestrus when there is no endometrial oedema, the uterine lumen can be seen as a thin, sometimes fragmented, white line and uterine layers are easily seen. In both stages of the cycle, myometrial contractions can be seen as undulations of the ventral and dorsal aspect of the uterine body and as coordinated wave-like motion of the endometrium. These wave-like movements appear to be changes in the length of the horizontal and vertical axes, following the contraction and relaxation patterns of the outer longitudinal and inner circular muscle layers. Electromyographic studies have suggested that uterine electrical activity, which is closely related to mechanical uterine activity (Toutaine *et al.*, 1983), is markedly more synchronized in oestrus than in dioestrus (Troedsson *et al.*, 1993a). For the purpose of this thesis, the term uterine contractile activity (UCA) was used to describe both synchronous and uncoordinated uterine activity. Uterine spasm, as seen after intravenous administration of ecbolic drugs, is accompanied by maximal uterine contraction that results in a sudden decrease of UCA and therefore is depicted by low UCA scores. The complete cessation of UCA at uterine spasm is followed by a gradual relaxation-contraction pattern. The amplitude then gradually increases until it returns to normal. In the present study, UCA was not evaluated for direction or frequency of peristaltic movement. Uterine contractility has been scored from 0 to 4 (Cross and Ginther, 1987; Cross and Ginther, 1988) or 1 to 5 (Griffin and Ginther, 1990) in the mare, and from 0 to 2 in women (Birnholz, 1984). For the purpose of this study, however, this method was modified to a scale of 1 (minimum) to 10 (maximum), which gave a higher scoring flexibility to the observer.

One minute segments of recorded UCA were evaluated separately. Two-dimensional motion of endometrial tissue reflectors, characteristic of the ultrasonographic uterine echotexture, was evaluated. Scoring was based on the visual evaluation of endometrial motion. Horizontal "wave" motion was observed directed both towards the uterine fundus and cervix, although direction and speed of such motion was not evaluated. Interestingly, in very active uteri, focal endometrial motion was observed. In such cases, a circular and very localised wave pattern could be simultaneously seen at different sites.

Scores between 0 and 2 were given either when there was no activity or when it was very low and the motion observed on screen was mostly due to the underlying intestinal peristaltic motion or the movement of the animal or the operator. Low scores were also given after OT injection when the uterus went into spasm and UCA was therefore minimised. Uterine spasm was differentiated from low UCA by the part of the screen that was covered by the uterus. The vertical uterine axis was shorter at the time of uterine spasm whereas when there was low UCA, the vertical uterine axis remained unaffected.

Scores between 3 and 6 were given to different levels of UCA observed which usually involved actively mostly the horizontal uterine axis. However when scores between 7 and 10 were given, both horizontal and vertical uterine axes tended to expand and shorten periodically thus forming a "wave" motion demonstrating strong uterine peristalsis.

Chapter 3

Uterine contractility is necessary for the clearance of intrauterine fluid, but not bacteria, after bacterial infusion in the mare

Introduction

Endometritis is the commonest cause of subfertility and the third most common medical problem in the mare (Traub-Dargatz *et al.*, 1991). Endometritis in the mare has recently been divided into four broad categories: sexually transmitted disease, chronic infectious endometritis, persistent mating-induced endometritis (PMIE) and chronic degenerative endometrosis (Troedsson *et al.*, 1995a; LeBlanc, 1997a). Susceptibility to PMIE is defined as the inability of the oestrous mare to clear intraluminal fluid accumulations within 12 to 48 hours after breeding (Katila, 1995; LeBlanc *et al.*, 1995c; Troedsson, 1997). The primary mechanism responsible for persistence of fluid appears to be impaired physical clearance of the uterus. It has been shown that mares with PMIE have reduced electrical myometrial activity in response to intrauterine bacterial challenge (Troedsson *et al.*, 1993b), are less able to clear the uterus of radiocolloid (LeBlanc *et al.*, 1994a) and other markers (Evans *et al.*, 1987; Troedsson and Liu, 1991), possibly have lymphatic dysfunction (LeBlanc *et al.*, 1995c) and have visibly lower uterine motility as determined by ultrasound scanning (Nikolakopoulos and Watson, 1997a – see chapter 6), than genitally normal mares. A recent study has shown that administration of phenylbutazone to block prostaglandin $F2\alpha$ synthesis, one of the ecbolic hormones, impeded clearance of radiocolloid from the uterus (Cadario *et al.*, 1995). In order to further understand the pathogenesis of PMIE it is important to demonstrate that impairment of uterine contractility can convert a genitally normal mare into a mare which accumulates fluid after intrauterine challenge.

In the present study clenbuterol, a β_2 agonist, was used to reduce the activity of the myometrium. $Beta_2$ agonists tend to inhibit contractile activity of the uterus rather than cause relaxation (Shapland *et al.*, 1981) and act as antagonists to the ecbolic effects of both oxytocin and prostaglandin $F2\alpha$ (Engelhardt, 1976). The most important action of β_2 agonists is to close the gap junctions between myometrial cells thereby decreasing the ability of action potentials to propagate between cells. Clenbuterol is widely used in

veterinary practice as a broncholytic (Zerobin and Kuendig, 1980; Engelhardt, 1976) and tocolytic (DeNooij, 1984; Greene, 1981) drug and has been used experimentally to suppress uterine contractility during early pregnancy in mares (Ginther, 1985). In the present study the effect of clenbuterol on uterine contractility, was firstly investigated and then went on to determine whether clearance of experimentally-induced intrauterine infection was impaired in genitally-normal oestrous mares treated with clenbuterol.

Materials and Methods

Animals

Five mares, weighing 320 to 500 kg and aged 4 to 11 years, were classified as resistant to PMIE according to their reproductive history of high fertility, ability to clear introduced intrauterine infection within 48 hours from the time of natural service and endometrial biopsy scores of 1 to 2A. None of the mares had any detectable intrauterine fluid accumulations prior to any of the experiments.

Experiment 1

When the mares (n=5) were detected in oestrus by teasing positively to a stallion, with an ovarian follicle greater than 30 mm diameter and uterine oedema, uterine activity was monitored ultrasonographically and recorded continuously on a video cassette recorder from 5 minutes before until 10 minutes after the administration of clenbuterol (1μg/kg Planipart, Boehringer Ingelheim Ltd., Bracknell, Berks; iv). Uterine activity was then monitored for 5 minutes every 2 hours for the following 8 hours. One minute segments of the videorecorded uterine activity were graded in a modified scale of 1 (minimum) to 10 (maximum) as described previously (Cross and Ginther, 1987; Cross and Ginther, 1988) (see Chapter 2).

Experiment 2

In order to extrapolate volume of intrauterine fluid with ultrasound images of fluid, the mares (n=4) were given an intrauterine infusion of 6 different volumes (20, 40, 60, 80, 100, 250 ml) of sterile phosphate buffered saline (PBS) during oestrus. The uterus was scanned to verify that all of the fluid went into the same pocket and the least possible pressure was applied to the uterus by the operator. On each occasion the area of the anechoic intrauterine fluid was calculated, within 2 minutes from infusion, by multiplying the maximum width and height from three different images of the two-dimensional ultrasonographic image and the mean calculated. For comparison, the mean diameter of the fluid in the uterus was calculated from the height and width of the anechoic image. Ultrasonographic measurements were plotted against volume infused.

Experiment 3

When mares (n=5) teased positively to a stallion, had ultrasonographic evidence of uterine edema, and a 35 mm ovarian follicle, they were injected intravenously with 2,400 IU hCG (Chorulon, Intervet, Cambridge, UK) to induce ovulation within 36 to 48 hours (Duchamp *et al.*, 1987). On the next day the mares were given an intrauterine infusion of a bacterial inoculum (40 ml). The tail was wrapped in a plastic sleeve, the rectum was evacuated manually, and then the genital area was washed three times with tamed povidone iodine solution and dried. The inoculum was infused via a sterile insemination pipette guided by a sterile gloved hand. Forty-eight hours after infusion, the reproductive tracts of the mares were examined by transrectal ultrasonography for the presence of intrauterine fluid. The ovarian findings were recorded and quantity of uterine fluid was extrapolated from the graph generated in experiment 2 (Fig. 3.1). The uterus was then flushed as described below. The procedure was repeated at a subsequent

estrus. This time an indwelling cannula, was placed in the jugular vein and clenbuterol (1μg/kg) was administered intravenously, 15 minutes before, and every 8 hours after the bacterial infusion for the next 48 hours until the uterus was flushed. An interval of 14 to 362 days (mean = 88.4±66.5 days) elapsed between the two treatments.

Uterine Flush

See Chapter 2

Preparation of the Inoculum

Streptococcus zooepidemicus was isolated from the uterus of a mare with acute endometritis and the bacteria were stored at -70°C in brain-heart infusion broth (BHIB), containing 10% glycerol. On the day before infusion, an aliquot was thawed, inoculated into BHIB and incubated overnight at 37°C. The bacteria were then washed three times in sterile phosphate buffered saline (PBS, pH 7.0) and resuspended at 750 x 10^6 colony forming units/ml by calibration of optical density. A sample of the inoculum was cultured at the time of each infusion to check the purity of the culture. Immediately prior to infusion, 1 ml of the washed bacteria was placed in 40 ml of sterile PBS.

Statistical Analysis

Uterine motility scores before and after clenbuterol administration in experiment 1 were compared using a paired t-test. The degree of correlation between area and diameter of intrauterine fluid and volume of infused fluid in experiment 2 were analyzed using a Spearman's rank test and its associated 95% confidence interval. Measurements made from the uterine flushes of the control and the clenbuterol-treatment group were compared using a paired t-test.

Results

Experiment 1

Uterine motility prior to the administration of clenbuterol ranged from grade 6 to 8 (Fig. 3.1). Within the first 15 minutes period after the intravenous administration of clenbuterol, uterine motility decreased significantly ($P<0.05$) and resumed former levels between 6 and 8 hours later.

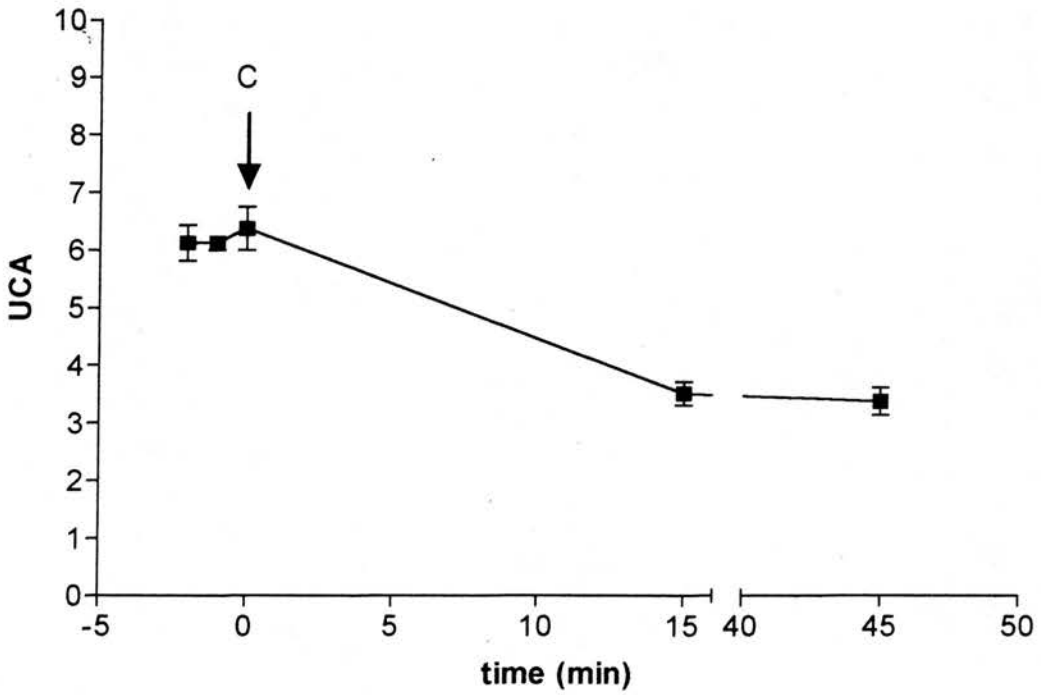

Figure 3.1. Uterine motility score, as detected by ultrasonography, before and after the administration of clenbuterol (C).

Mean (±SEM) anechoic areas of fluid in the uterus associated with each volume of infused fluid are shown in Fig. 3.2. Immediately after the infusion, it was seen that fluid volumes up to 100 ml had the tendency to form a single pocket of fluid located around the horn-body junction. However measurements were not obtained from the mares after infusion with 250 ml since the fluid dispersed into several locations in the uterus. Area of the fluid was well correlated with volume infused (r=0.975), whereas the mean diameter of the non-echogenic fluid image was only poorly correlated with infused volume (r=0.465).

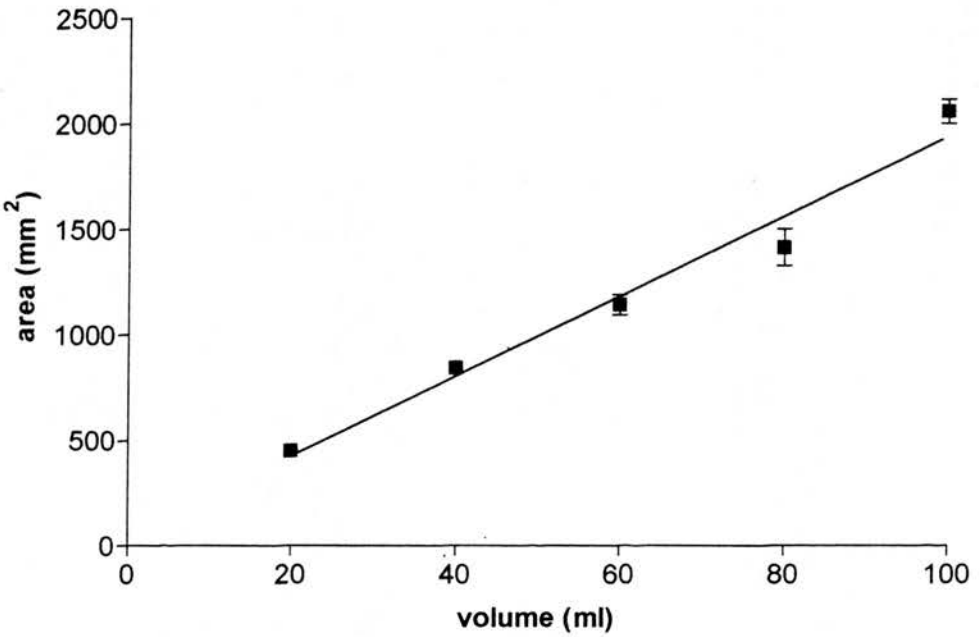

Figure 3.2. Ultrasonographic measurements of area (mm ^{2}) calculated after infusion of different volumes of fluid into the uterus of the mare (r=0.975).

Experiment 3

Pure growth of *Streptococcus zooepidemicus* was isolated from all samples of the cultured inoculum. All mares had ovulated by the time of the uterine flush, 48 hours after infusion of the bacterial inoculum. None of the mares had any uterine fluid accumulation during the control cycle but all mares accumulated fluid during the treatment cycle. Volumes were extrapolated from ultrasonographic area measurements and are shown in Table 3.1.

Table 3.1. Gross, cellular and bacteriological findings 48h after bacterial inoculation for the clenbuterol-treated (C) and the non-treated (N) group of mares.

Mare	Volume of Uterine fluid ml		Quality of recovered fluid [a]		Cell number recovered x10^4/ml		Bacteria isolated [b]		Percentage of Neutrophils	
	N	C	N	C	N	C	N	C	N	C
1	-	77	1	1	11.3	6	-	+++	7	61
2	-	84	2	2	32	8	+	-	75	70
3	-	64	2	3	79	430	+*	+++	78	97
4	-	46	2	4	109	185	++	-	99	97
5	-	54	1	3	7	110	-	-	39	75
Mean± SEM		65±7	1.6± 0.2	2.6± 0.5	56.7± 20.5	147.8 ±78.3			59.6± 16.3	80± 7.3

[a] Quality of recovered fluid was graded on a scale of 0 (crystal clear) to 5 (pus).
[b] Bacteria isolated in culture (- negative, + under 10, ++ 10-100, and +++ more than 100 colonies). Only *S. zooepidemicus*, was isolated except in one mare (*) where *E.coli* was isolated.

The volume of recovered uterine fluid was approximately the same (55±3.7 ml) as the volume infused, even if there was already fluid in the uterus. Although cloudiness of recovered fluid tended to be greater after clenbuterol treatment, this failed to reach significance (P=0.09). There was no significant difference between the control and treatment cycles in number of cells and percentage of neutrophils recovered in the uterine flush (Table 3.1). A heavy growth of *S. zooepidemicus* was isolated from 2 of

the mares in the clenbuterol-treatment cycle. Two mares from the control treatment cycle had fewer than 10 colony forming units which was probably due to contamination (Hinrichs *et al.*, 1988) and one had a moderate growth of *S. zooepidemicus* (Table 3.1). Within 36 hours after the last clenbuterol treatment (day 3 postovulation) none of the treated mares had detectable intrauterine fluid.

Discussion

Mares which retain uterine fluid for more than 12 hours after mating are considered to have PMIE (Troedsson, 1997). It was shown for the first time that inhibiting myometrial activity in genitally-normal mares results in the presence of intrauterine fluid 48 hours after bacterial challenge. Thus resistant mares were effectively converted into susceptible mares.

In our mares ultrasonographic monitoring showed that uterine smooth muscle activity decreased significantly within 15 minutes of clenbuterol administration and the effect lasted for 6 to 8 hours. This finding is similar to reports in the cow where uterine motility decreased within 10 to 20 minutes (DeNooij, 1984), and parturition was postponed for 5 to 8 hours (Greene, 1981). Although, as predicted, uterine activity was not abolished by administration of clenbuterol, it was substantially reduced. Clenbuterol is currently used at a dosage similar or higher (0.8-3.2 μg/kg b.i.d.) than that used in the present study, for long periods of time in the treatment of chronic obstructive pulmonary disease (COPD) (Lavoie 1997). It is possible that by interfering with uterine smooth muscle activity such therapy may predispose brood mares to the accumulation of intrauterine fluid and cause fertility problems.

The presence of a small volume of anechoic intrauterine fluid during oestrus, in the absence of microbiological and cytological signs of acute inflammation, is generally not of inflammatory origin (Allen and Pycock, 1988; Reilas *et al.*, 1997; Pycock and

Newcombe, 1996b). However, the detection of intrauterine fluid in the uterus of the mare after breeding is usually a sign of uterine inflammation and is related to reduced fertility (Adams *et al.*, 1987). In the quantification and scoring of intrauterine fluid accumulations in the mare, image size measurements (Adams *et al.*, 1987; Pycock and Newcombe, 1996b), fluid echogenicity (McKinnon *et al.*, 1987) and percentage of cases with intrauterine fluid accumulations in different uterine locations (Jones, 1995) have been employed. In the present study, the form of the created pocket was approximately rectangular or ellipsoidal depending on its position within the uterus and therefore its area could be calculated from the multiplication of the two readings. These area measurements correlated well with volume infused and allowed us to extrapolate volume of accumulated intra-uterine fluid. When the volume of infused fluid was correlated with the mean diameter of two readings (height and width) as it is often done in practice, statistical analysis showed only poor correlation. In our study volumes of intrauterine fluid greater than 100 ml were usually dispersed in multiple pockets over a greater area of the uterine body and horns.

In clinical cases of PMIE the causes of the presence, distribution and retention of uterine fluid are multifactorial. Poor perineal conformation, fibrosed cervix, multiparity and age, among others (Cadario *et al.*, 1995), cause structural changes to the uterus that impair uterine contractility and lymphatic drainage, compromising uterine clearance. In older, multiparous, mares, a more flaccid and relaxed uterus extending over the brim of the pelvis is more likely to evacuate intrauterine fluid accumulations at a much slower rate than in maiden mares (LeBlanc *et al.*, 1998). In the present study young reproductively sound, maiden mares were used, and therefore specifically addressed the role of uterine motility in expelling intrauterine fluid without the presence of confounding conformational factors.

Low-volume uterine flush as employed in this study is a technique that allows the sampling of a larger surface of the endometrium than the endometrial swab (Ball *et al.*, 1988). The degree of cloudiness of the recovered intrauterine fluid has been positively

correlated with the amount of white blood cell infiltration and is an indication of the degree of inflammation (McKinnon *et al.*, 1987). In our study, cell numbers tended to be higher in the clenbuterol-treated group but this difference failed to reach significance. It is probable that number of cells per ml of recovered intrauterine fluid in the clenbuterol-treated group was diluted by the presence of existing intrauterine fluid prior to flushing. Failure to compensate for existing uterine fluid has been described as a problem in calculating the numbers of cells in uterine flushes (Heap 1962).

Resistant mares usually have very few intrauterine neutrophils when the uterus is free from inflammation (Brook, 1985). The induction of uterine inflammation by bacterial inoculation causes neutrophil infiltration that peaks at about 6 hours after the introduction of bacteria into the uterus (Williamson *et al.*, 1987; Watson *et al.*, 1988b). In the present study percentages of neutrophils in uterine lavage fluid were high in both groups, which is similar to the findings of chapter 6 (Nikolakopoulos and Watson, 1997b) where neutrophilia was still present 48 hours after artificial insemination in both resistant and susceptible mares.

Response to artificial insemination is probably the uterine challenge that best classifies a mare as a resistant or susceptible to PMIE as will be shown in chapter 6. Equine spermatozoa are chemotactic for neutrophils *in vitro* (Troedsson *et al.*, 1995a) and it has been shown that artificial insemination results in an inflammatory response in both susceptible and resistant mares, despite the addition of antibiotic to the extender (see chapter 6). This emphasizes the importance of sperm in the antigenic challenge *in vivo* (Kotilainen *et al.*, 1994). However, it is inevitable that bacterial contamination will result from breaching the mare's cervix, and the intrauterine infusion of a bacterial inoculum in the present study provided a controlled and uniform stimulus which has been widely used in the past (Troedsson *et al.*, 1993b; LeBlanc *et al.*, 1989; McDonnell and Watson, 1992). The numbers of bacteria recovered from the lavage fluid from the non-treated group were low to moderate. Only one mare in the control cycle had a significant number of bacteria in recovered uterine fluid. Two mares had fewer than 10

colonies which can be regarded as insignificant (Hinrichs *et al.*, 1988). However, perhaps more interestingly, heavy growth of *S. zooepidemicus* was observed in only two mares from the clenbuterol-treated group. In chapter 6, it was shown that not all susceptible mares are infected with bacteria after intrauterine challenge and the findings in this chapter suggest that PMIE is caused more by a defect in uterine contractility than by a deficit in antibacterial properties of the uterus. The results presented here show that high uterine motility is not necessary for elimination of uterine bacteria and also that intrauterine fluid can accumulate in the absence of infection.

The rapid timescale of bacterial clearance suggests that non-specific antibacterial mechanisms, such as the antibacterial properties of uterine fluid and neutrophil phagocytosis are responsible for elimination of bacteria, rather than specific immunological mechanisms. The results of our study suggest that the widespread use of antibiotic infusion in mares with PMIE (Pycock and Newcombe, 1996a) may not be justified. Rather the use of ecbolics alone possibly combined with uterine lavage between 6 and 12 hours after breeding mares with PMIE (LeBlanc and Asbury, 1994; LeBlanc, 1994; Troedsson, 1997), should be encouraged.

Uterine cellular and bactericidal mechanisms appear to be dysfunctional during the postovulatory period because of the suppressive effects of progesterone on uterine defense mechanisms, uterine contractility and cervical closure (Evans *et al.*, 1986; LeBlanc *et al.*, 1989; Watson, 1987c; Watson *et al.*, 1987c). However, within 36 hours of the low volume uterine flush and the end of the clenbuterol treatment, all mares in the present study had cleared intrauterine fluid accumulations. Therefore even in the presence of increasing concentrations of progesterone, once uterine contractility returned to normal, intrauterine fluid accumulations were evacuated despite the negative influence of progesterone.

In conclusion, genitally-normal estrous mares successfully expelled intrauterine fluid accumulations by 48 hours after bacterial challenge whereas reduction of uterine

contractility in the same mares resulted in accumulations of intrauterine fluid. It appears therefore that clenbuterol converted the resistant uterus to a susceptible uterus by inhibiting myometrial activity. Also because 3 of the 5 mares with intrauterine fluid accumulations eliminated bacteria, it can be concluded that bacteria can be cleared either by non-specific antibacterial defense mechanisms and/or that only baseline uterine motility is necessary for bacterial elimination. These results therefore strongly support data from other workers showing that impaired uterine contractility contributes to persistent mating-induced endometritis in mares.

In this chapter, the importance and function of UCA in the elimination of intrauterine fluid accumulations was demonstrated. Furthermore, it was shown that despite the ability of the humoral and cellular or other non-specific defence mechanisms to partially neutralize the bacterial infection the role of UCA is essential for the physical clearance of intrauterine fluid and cellular debris. It is well established that ecbolic hormones control UCA to a great extent. However, there is still no detailed information on the dynamics of ecbolic hormone release at the time of application of different stimuli, such as breeding and other reproductive events, and consequently their effect on UCA. In the next chapter, the effect of common reproductive events such as natural service, teasing and genital manipulations, on the release of OT and $PGF_{2\alpha}$ will be investigated.

Chapter 4

Oxytocin and $PGF_{2\alpha}$ release around teasing, natural service and associated events in the mare

Introduction

Concentrations of plasma oxytocin (OT) in cycling mares have variously been reported to be highest at oestrus (Burns *et al.*, 1981) or dioestrus (Tetzke *et al.*, 1987), or to remain at low levels throughout the cycle (Stevenson *et al.*, 1991). In other species release of OT can be elicited at oestrus by mating and presence of the male (Fox and Knaggs, 1969; McNeilly and Ducker, 1972; Gilbert *et al.*, 1991) and these high concentrations of OT may serve to facilitate gamete transport (Wathes, 1984; Carmichael *et al.*, 1987; Gilbert *et al.*, 1992). The pattern of OT release at mating appears to be highly species-dependent. Investigators have failed to detect any rise in OT levels around mating in cows (Schams *et al.*, 1982). By contrast, a rise in OT levels at the time of mating has been observed in women, sows, goats and mares (Fox and Knaggs, 1969; McNeilly and Ducker, 1972; Todd and Lightman, 1986; Claus and Schams, 1990; Alexander *et al.*, 1995). Results in ewes and rabbits are contradictory with some workers finding an increase in circulating OT concentrations at mating (Debackere *et al.*, 1961; Fuchs *et al.*, 1981a; Gilbert *et al.*, 1991) while others failed to observe any changes (Sharma and Chaudhury, 1970; Garcia-Villar *et al.*, 1985). In some species the physical stimulus of coitus is thought to be a relatively minor component in stimulating the release of OT (McNeilly and Ducker, 1972) and OT peaks may be provoked by the sensory and psychic stimuli occurring at the time of mating (Campbell and Petersen, 1953; McNeilly and Folley, 1970; McNeilly and Ducker, 1972; Mattioli *et al.*, 1986). In the mare, mating is a complex combination of sensory, psychic and mechanical stimuli involving teasing, mounting, intromission and ejaculation by the stallion. It is known that teasing stimulates OT peaks in the mare at mating (Alexander *et al.*, 1995) but there is only limited information on the pattern of OT release (Walmsley 1963; Alexander *et al.*, 1995).

Prostaglandin F2α (PGF2α) release is high in late dioestrus in mares, in association with luteolysis (Neely *et al.*, 1979b; Goff *et al.*, 1984). Release of PGF2α can also be

stimulated at other stages of the cycle by OT (Goff *et al.*, 1987) and there is a positive correlation between plasma OT concentrations and $PGF2\alpha$ release in non-pregnant mares (Sharp *et al.*, 1997). Therefore any increase in OT at mating in mares might be accompanied by release of $PGF2\alpha$. Both $PGF2\alpha$ and OT have been implicated as ecbolic agents in mares (Troedsson *et al.*, 1995e). Increases in concentrations of these two hormones around the time of mating may therefore be important not only in gamete transport, but in stimulating uterine contractile activity after mating which serves to evacuate collections of inflammatory uterine fluid and cellular debris (Hughes and Loy, 1975).

In the present study, the release patterns of the ecbolic hormones OT and $PGF2\alpha$ were determined, at the time of mating in the mare and some hours following mating when clearance of uterine contamination may occur. The effect of a range of stimuli associated with mating on OT and $PGF2\alpha$ release were also investigated.

Materials and methods

Animals

Eleven fertile mares, aged 5 to 15 years and weighing between 350 and 480 kg, were used. These mares had been classified as genitally normal, according to their reproductive history, negative endometrial cytology and culture, and endometrial biopsy scores of 1-2A (Kenney *et al.*, 1986). Oestrus was detected by teasing with a stallion, combined with transrectal ultrasonographic examination of the genital tract. When the mare responded positively to teasing and uterine oedema was present, with a follicle of at least 35 mm present on the ovaries, the mare was considered to be in oestrus. Ovulation was detected ultrasonographically by the disappearance of the follicle and the presence of a corpus haemorrhagicum on the ovary. Day of ovulation was designated as day 0. A stallion with good libido was used in experiments 1 and 2.

Experimental Procedure

The same blood sampling protocol was used in all experiments. On the day of the experiment an indwelling cannula (13 gauge, Presidio Medico, Ecouen, France) was placed in the jugular vein aseptically under local anaesthesia. Blood samples were collected at 2 minutes intervals for 30 minutes before, during, and one hour after the application of the stimulus, and then for another hour, at 5 minutes intervals.

Four different stimuli were applied. In experiment 1, oestrous (n=5) and dioestrous (day 7; n=5) mares were teased by the stallion for 6 minutes (3 samples) in teasing stocks. The stallion was allowed to nuzzle and nip the mare. In experiment 2, oestrous mares were teased as in experiment 1, but in this case teasing was followed immediately by mating (n=5). In experiment 3 the genital tracts of oestrous mares (n=4) were manually manipulated to simulate the individual stimuli associated with mating. All stimuli applied were exaggerated in duration in order to intensify the response to the particular stimulus. The mares' external genitalia were actively massaged for 6 minutes (3 samples), while washing three times with tamed povidone iodine solution, after which the operator inserted a sterile gloved lubricated hand into the vagina, moving it carefully palindromically for 4 minutes (2 samples), distending the vaginal walls without coming into contact with the cervix. The cervix was then massaged and manipulated for 2 minutes (1 sample). In experiment 4, the mechanical effect that the introduction of fluid into the uterus and/or uterine distension has on hormonal release in oestrous mares (n=5) was determined by intrauterine infusion of 500 ml PBS (pH 7.0). Additional blood samples were collected every 15 minutes between 16 and 18 hours after mating for measurement of PGFM concentrations to determine whether the high uterine myoelectrical activity reported at this time after bacterial infusion (Troedsson *et al.*, 1993b) is due to the release of prostaglandins.

Sample Handling

See Chapter 2.

Oxytocin assay

See Chapter 2.

PGFM assay

See Chapter 2.

Statistical Analysis

Mean baseline hormonal concentrations were calculated from the average of the values obtained prior to the application of the stimulus (Time 0) and mean hormonal concentrations were calculated for every 30 minutes interval thereafter. Concentrations in samples below the detection limit of the assays were designated as equivalent to the detection limit of the respective assay. In the case of teasing and manipulation of the genital tract, mean stimuli values for both hormones were obtained from the samples corresponding to the time of the different stimuli application. During natural service and uterine infusion, mean OT values were obtained from the samples corresponding to the mean OT concentrations of the peak immediately after the application of the stimulus until OT concentrations returned to baseline levels.

Mean baseline OT and PGFM concentrations from all experiments were compared using one way ANOVA test. Mean baseline values and mean stimuli values for both OT and PGFM, for each experiment, were compared using a two sample t-test. In experiment 3, mean stimuli values for both OT and PGFM were compared with mean baseline values separately for each stimulus. The difference in magnitude of OT responses between different stimuli was compared using one-way ANOVA tests.

Because of the pulsatile nature of OT release and its short half-life, responses were assessed for individual mares. A mare was considered to have a positive OT response when the mean concentration of the peak immediately after the application of the stimulus exceeded the mean baseline concentrations + 2 x SD. Mean PGFM concentrations were calculated for each mare as above (30 minutes intervals) and a positive response was recorded when the increase for each mare, at any 30 minutes interval after the stimulus application, exceeded the mean baseline concentrations + 2 x intra-assay coefficient of variation.

Results

Experiment 1

For the evaluation of responses to teasing, teasing periods before mating were also included. There was no significant difference ($P>0.1$) either between the number of mares responding to teasing with increased OT release in oestrus (6/10) and dioestrus (3/5) or in the magnitude of response. All oestrous teasings were accompanied by a display of oestrous behaviour such as posturing and clitoral winking, while during all dioestrous teasings the mares demonstrated aggressive behaviour and rejected the stallion. Teasing caused a significant ($P<0.05$) increase in OT concentrations in both oestrus and dioestrus (Table 4.1). In both dioestrous and oestrous teasing periods, except those followed by mating, mean OT concentrations declined to baseline values within 30 minutes (Table 4.1). On three occasions an OT peak was observed immediately prior to the initiation of teasing, coinciding with the entrance of the stallion into the mare's visual field (Figures 4.2.b and 4.4.b).

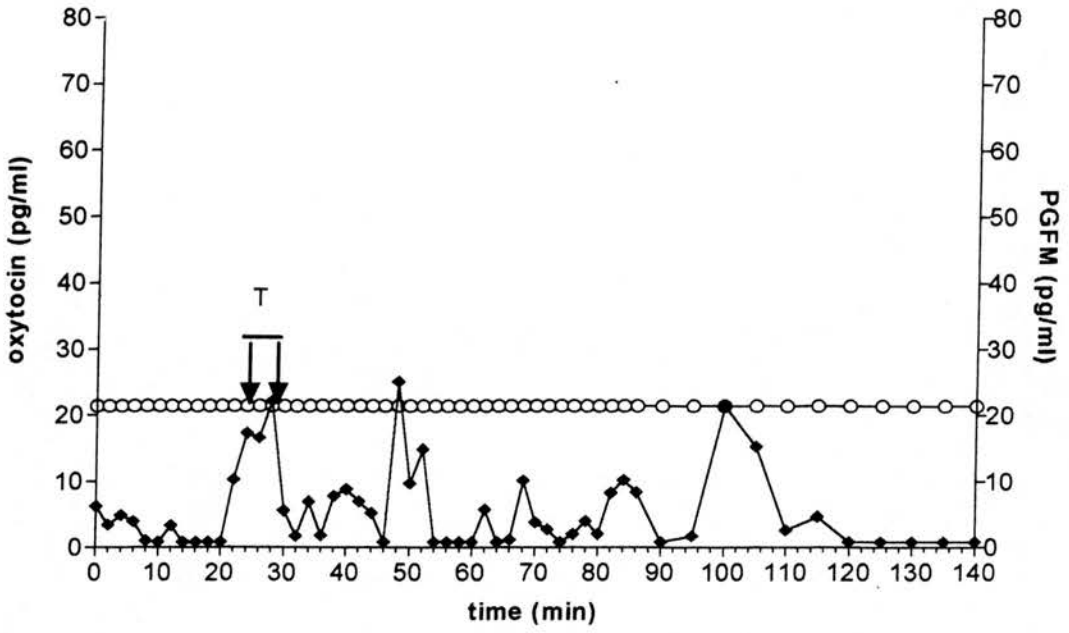

Figure 4.1. Oxytocin (♦) and PGFM (O) levels around dioestrous teasing (T).

Although teasing had no significant effect on mean PGFM concentrations in oestrus (Table 4.1), elevations were observed in 2 of the 5 sampling periods after teasing (Figures 4.2.b and 4.3.b). None of the dioestrous mares released significant amounts of $PGF2\alpha$ at teasing (Table 4.1).

Experiment 2

All 5 mares had a positive OT response during natural service and mean stimuli values were significantly higher ($P<0.05$) than baseline values (Table 4.1). Furthermore in 2 mares in which false mounting occurred without intromission prior to successful mating, an oxytocin peak was observed (Figures 4.2.a and 4.3.a). Mean OT concentrations after mating declined to baseline values within 30 minutes after the application of the stimulus.

Table 4.1. Mean (± SEM) plasma oxytocin and PGFM concentrations, before, during (n=10) and after (n=5) oestrous teasing episodes, dioestrous (D 7) teasing episodes (n=5), natural service (n=5), manipulation of the genital tract involving massage of the external genitalia [1], distension of the vaginal walls [2] and cervical manipulation [3] (n=4), and distension of the uterine walls by infusion of 500 ml PBS (n=5). The asterisk (*) indicates significant difference (P<0.05) from baseline concentrations.

	Experiment 1				Experiment 2		Experiment 3		Experiment 4	
	Tease (Oestrus) n=10		Tease (D7) n=5		Natural Service n=5		Manipulation n=4		Distension n=5	
Time (min)	OT (pg/ml)	PGFM (pg/ml)	OT (pg/ml)	PGFM (pg/ml)	OT (pg/ml)	PGFM (pg/ml)	OT (pg/ml)	PGFM (pg/ml)	OT (pg/ml)	PGFM (pg/ml)
-30-0	3.2±0.4	22.5±1.1	7.5±2.7	21.5±0.1	1.7±0.6	33.7±7.6	5.5±0.7	22.7±1.3	7.9±1.6	21.4±0.0
Stimulus	22.6±7.0*	25.2±3.4	13.7±1.3*	21.4±0.0	12.4±10.3	28.4±6.6	40.9±29.8 *1	21.5±0.1 [1]	32.9±13.1*	21.4±0.0
					22.7±7.5*	33.4±8.9	190.3±61.4 *2	33.4±10.7 [2]		
	n=5						149.4±132.3[3]	124.4±88.5 [3]		
0-30	4.4±0.5	31.8±8.9	8.5±0.7	21.8±0.3	2.2±0.4	29.2±4.8	21.5±7.6*	196.7±145.7	10.8±3.3	22.0±0.6
30-60	2.6±0.5	27.8±6.3	7.7±0.9	21.9±0.5	1.6±0.6	26.0±2.9	9.3±3.1	59.4±22.3	9.4±3.3	27.3±5.9
60-90	2.9±0.7	23.0±1.6	7.4±0.8	21.6±0.1	1.2±0.2	25.9±2.7	5.8±2.8	31.0±5.6	7.9±2.7	25.8±4.4

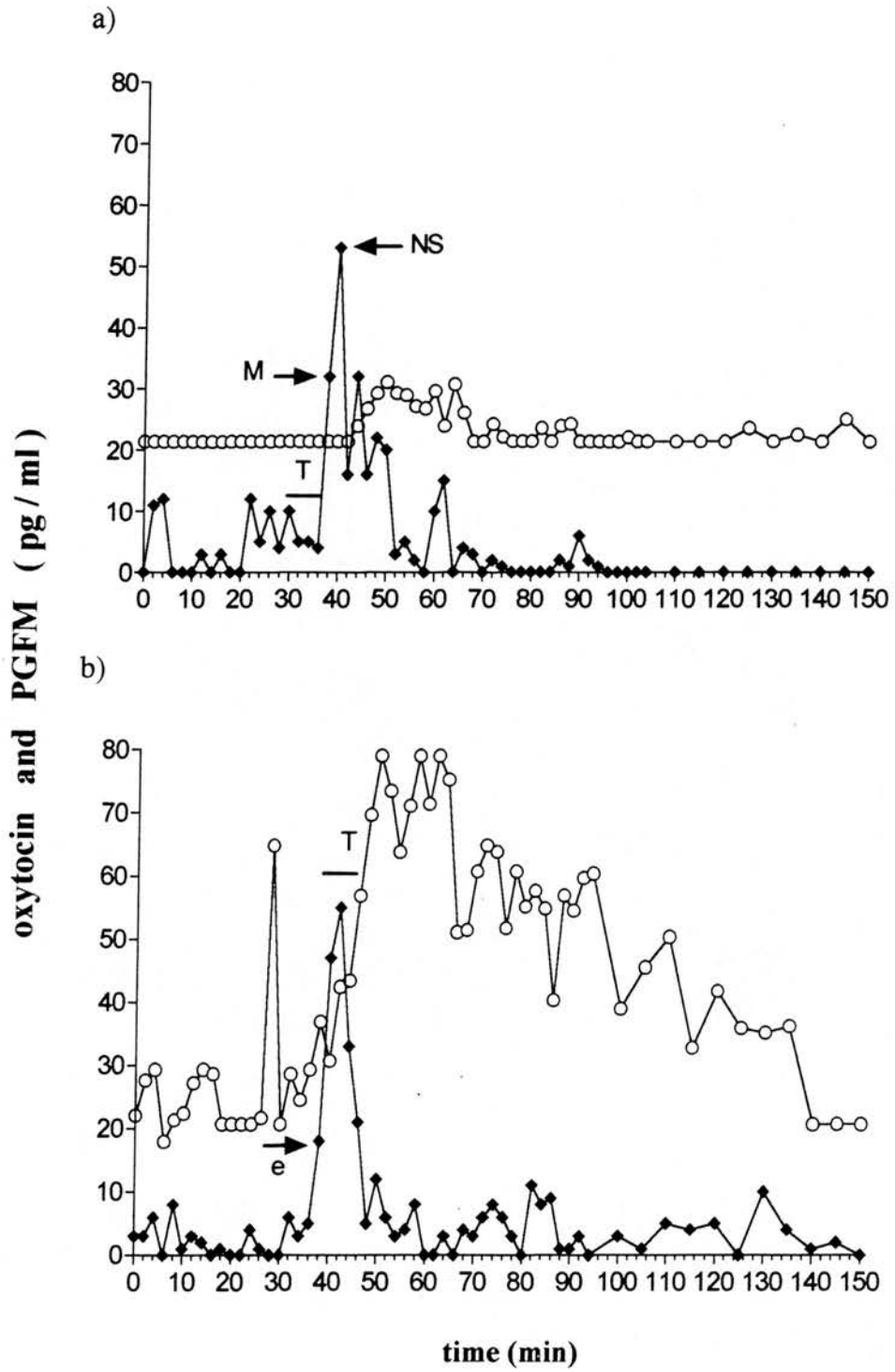

Figure 4.2. Oxytocin (♦) and PGFM (O) levels around natural service (NS) (a) and teasing (T) (b) in an oestrous mare. The entrance of the stallion in the visual field of the mare (e) and the false mount (M) are also noted.

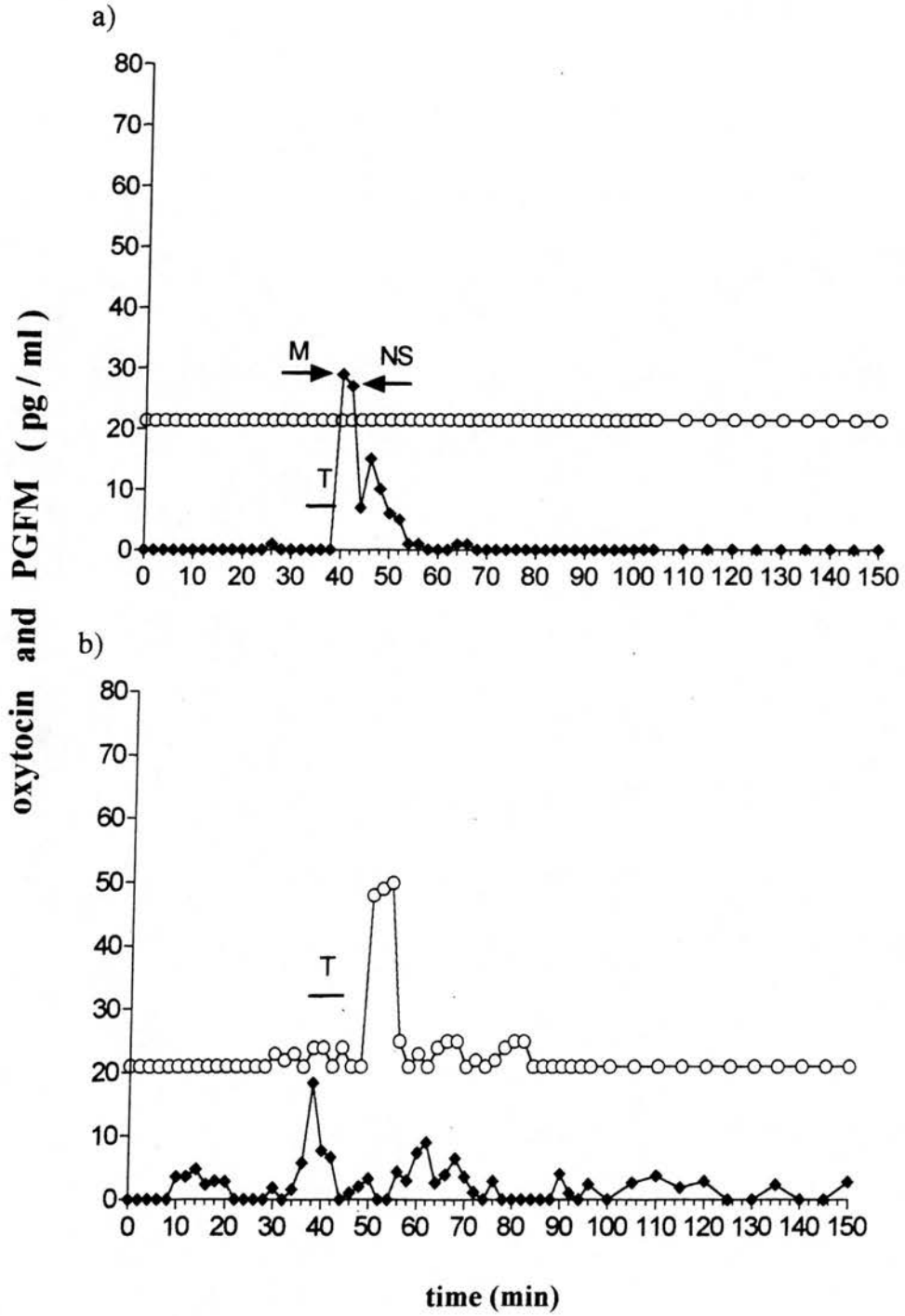

Figure 4.3. Oxytocin (♦) and PGFM (O) levels around natural service (NS) (a) and teasing (T) (b) in an oestrous mare. The false mount (M) of the stallion is also noted.

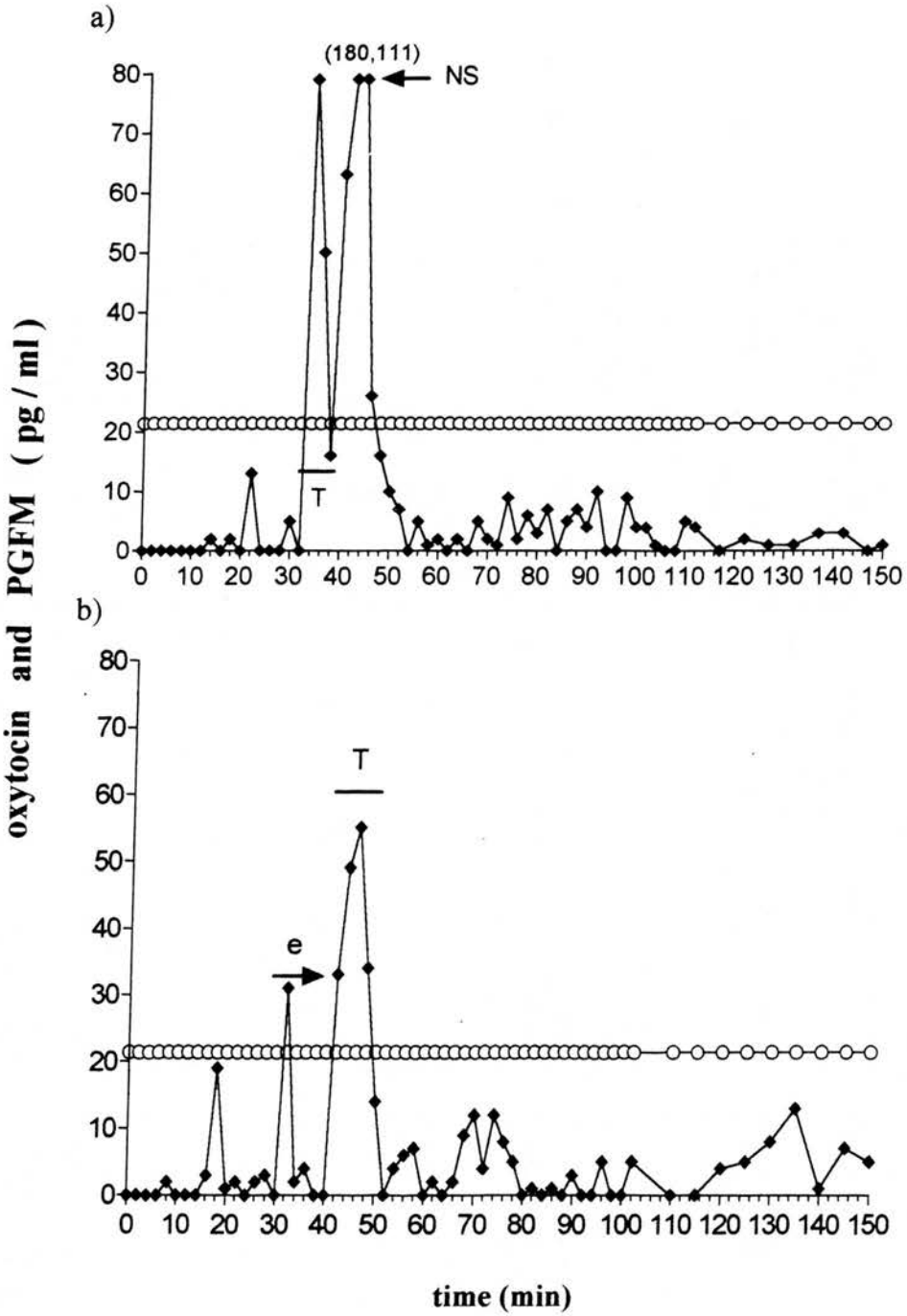

Figure 4.4. Oxytocin (♦) and PGFM (O) levels around natural service (NS) (a) and teasing (T) (b) in 3 of the oestrous mares. The entrance of the stallion in the visual field of the mare (e) is also noted.

Mating had no significant effect on mean PGFM concentrations (Table 4.1), but minor elevations were observed in 3 of the 5 mares after mating (Figure 4.2.a). There was no detectable PGFM in the samples collected 16-18 hours after natural service (data not shown).

Experiment 3

Manipulation of the genital tract consisted of three distinct stimuli to which the OT and PGFM response varied (Figure 4.5.). Active massage of the clitoris and external genitalia significantly ($P<0.05$) increased OT concentrations in 2 of 4 (50%) mares. All mares had a positive OT response to the distension of the vaginal walls, and cervical stimulation provoked a positive response in 3 mares (75%). Both distension of the vaginal walls and cervical stimulation had a significant ($P<0.05$) effect on mean OT concentrations.

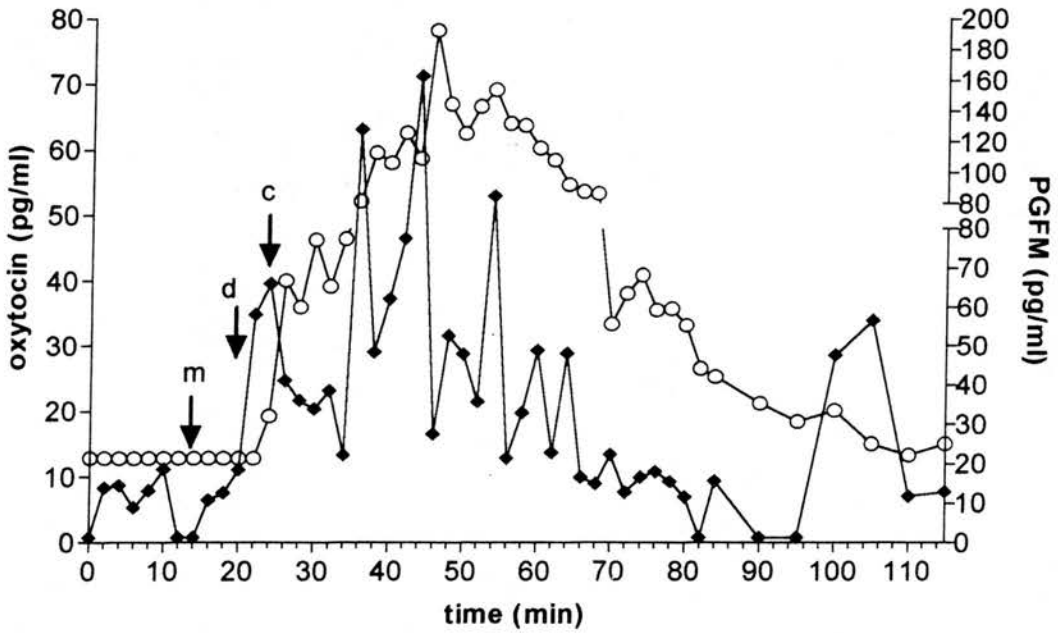

Figure 4.5. Oxytocin (♦) and PGFM (O) levels around manipulation of the genital tract in an oestrous mare. Active massage of the vulva and clitoris (m), distension of the vaginal walls (d) and cervical manipulation (c) are noted.

Only one mare responded positively to all three stimuli. Two of the 4 mares (50%) had detectable release of PGFM at the time of massage of the external genitalia, vaginal distension and cervical stimulation, and in only one of these mares did PGFM levels reach statistical significance when compared to mean baseline concentrations. In all mares, mean OT concentrations returned to baseline levels within 1 hour of the application of the stimuli (Table 4.1). Mean PGFM concentrations in the responding mare did not return to baseline levels by the end of blood sampling, 2 hours after the application of the stimuli (Table 4.1).

Experiment 4

The intrauterine infusion of 500 ml PBS provoked a positive OT response ($P<0.025$) in 3 of 5 (60%) mares (Figure 4.6). Mean OT concentrations returned to baseline levels within 1 hour from the time of infusion (Table 4.1). There was a positive PGFM response in only 1 mare (Figure 4.6).

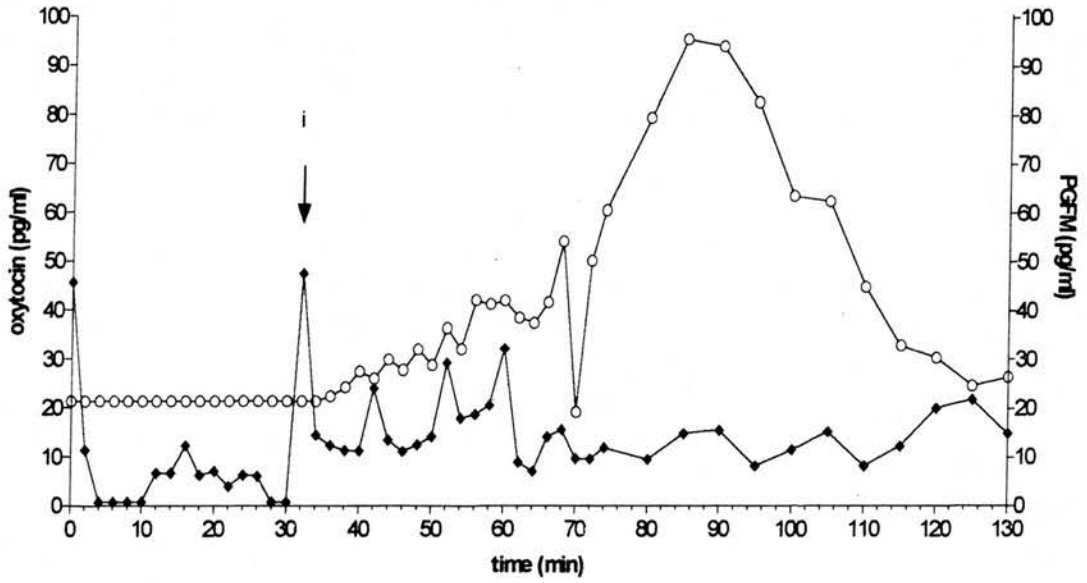

Figure 4.6. Oxytocin (♦) and PGFM (O) levels around intrauterine infusion of 500 ml PBS. The time of infusion (i) is noted.

The magnitude of OT response to the distension of the vaginal walls was significantly ($P<0.05$) higher when compared to the responses of all the other stimuli applied. None of the other stimuli applied were significantly different from one another as measured by OT and PGFM release.

Discussion

Pulsatility of OT secretion has been described in the ewe (Mitchell *et al.*, 1982), cow (Walters *et al.*, 1984) and mare (Tetzke *et al.*, 1987; Vanderwall *et al.*, 1998). Unlike ruminants (for review see Wathes, 1989), OT in the mare is released only from the posterior pituitary (Vanderwall *et al.*, 1998) and not from the ovaries (Stevenson *et al.*, 1991), although the uterus may contribute to local levels (Behrendt *et al.*, 1997; Watson *et al.*, 1997). There is little agreement on the amplitude and frequency of episodes of OT release in the mare or on concentrations at different stages of the cycle (Burns *et al.*, 1981; Tetzke *et al.*, 1987; Stevenson *et al.*, 1991; Sharp *et al.*, 1997). In the present study there was no significant statistical difference between oestrous and dioestrous (day 7) baseline OT concentrations.

The effect of central OT on memory, reproductive and maternal behaviours has been reported to be species-specific and dependent on concentrations of sex steroids (Caldwell *et al.*, 1989; Carter, 1992; de Wied *et al.*, 1993; Insel *et al.*, 1997). It has been suggested that OT initiates sexual behaviour in female rats, since OT infusion into the caudal ventromedial nucleus of the hypothalamus triggered displays of lordosis (Schumacher *et al.*, 1990) and increased tolerance of the female rat to tactile stimulation (Witt and Insel, 1990; Witt and Insel, 1991). It has been shown in other species that external stimuli such as olfactory (McNeilly and Folley, 1970; McNeilly and Ducker, 1972; Mattioli *et al.*, 1986), visual (McNeilly and Folley, 1970; McNeilly and Ducker, 1972), and tactile (Schams *et al.*, 1982) signals associated with teasing are directly responsible for the

release of OT. In the mare, posturing is an indicator of oestrus and is an indispensable part of mating behaviour. It is provoked by visual, tactile and acoustical stimulation during teasing (Veeckman and Oedberg, 1978). In the present study, although oestrous teasing was associated with sexually receptive behaviour in all mares, OT peaks were detected only in some mares. In agreement with the present study, Madill et al. (1998) reported that not all oestrous mares released pituitary OT in response to stallion-derived stimuli. It has been suggested that the observed peaks of OT secretion could act to reinforce signs of oestrus over the prolonged period of sexual receptivity in the mare (Alexander *et al.*, 1995) but results from this study would suggest that they are not prerequisite for the exhibition of oestrus. It is also possible however that peaks of OT were missed in the present study by sampling peripheral blood, as concentrations of OT are eight fold higher in blood collected from the intracavernous sinus (Vanderwall *et al.*, 1998). Exhibition of oestrus in the mare seems to be primarily dependent on the circulating concentrations of steroid hormones since, in our study, teasing at day 7 of dioestrus, when progesterone levels are high, provoked an OT response that was accompanied by rejection of the male. These observations support the concept that the steroid environment can modify responsiveness to somatosensory stimuli mediated by OT in the brain (Caldwell *et al.*, 1996).

The physical stimulus of coitus *per se* is a relatively minor component in stimulating the release of OT in the oestrous ewe (Bezlyudnik and Ambrosova, 1988), goat (McNeilly and Folley, 1970; McNeilly and Ducker, 1972) and cow (Schams *et al.*, 1982). By contrast, coitus-evoked OT release was reported in sows (Claus *et al.*, 1989) and in women (Fox and Knaggs, 1969; Carmichael *et al.*, 1987). It has been suggested that these inter-species differences are related to the degrees of physical stimulation caused by the male (Schams *et al.*, 1982; Claus *et al.*, 1989). The mating sequence in the mare includes a total mount time of 20 to 30 seconds with an insertion time before ejaculation of 10 to 15 seconds during which the stallion thrusts from 6 to 9 times (McDonnell, 1992). In the mare, the oestrous cervix is relaxed, permitting dilation by the greatly distended glans penis, thus allowing intrauterine deposition of semen. Therefore it might

be expected that OT would be elevated in the mare at mating. Walmsley (1963) detected a rise in plasma OT after mating in only 1 of 4 mares studied and Alexander and coworkers (1995) reported an elevation of OT levels in 2 of 3 mares in single samples collected within 5 minutes after mating. In our study all 5 mares showed substantial OT release around the time of mating in agreement with the results of Madill and coworkers (1998).

In two mares which had to be mounted twice by the stallion, the false mount was associated with release of OT on both occasions. Similarly in the cow (VanDemark and Hays, 1952) and the ewe (Lightfoot, 1970) mounting without intromission caused an immediate increase in the frequency and amplitude of uterine contractions possibly reflecting OT release. The release of OT at the time of mounting without intromission could reflect psychogenic release of OT or "stress". Elevated cortisol levels associated with mating behaviour (Rabb *et al.*, 1989) induce OT release (Antoni, 1986) and in this study might be involved in the OT release at the time of mounting.

Intromission and ejaculation are connected with significant tactile stimulation of the vaginal walls, cervix and the uterus (Todd and Lightman, 1986). Stimulation of the external genitalia and clitoris did not cause OT release in cows (Schams *et al.*, 1982) but self-stimulation provoked an OT response in women (Carmichael *et al.*, 1987). In our study, mechanical stimulation of the external genitalia and clitoris provoked OT release in two mares. Vaginal distension caused OT release in the cow (Schams *et al.*, 1982), ewe (Debackere *et al.*, 1961; Roberts and Share, 1969; Flint *et al.*, 1975; Kendrick *et al.*, 1991), goat (Blank and De Bias, 1977), and rats (Dreifuss *et al.*, 1976) and cervical manipulation has been reported to cause OT release in the ewe (Debackere *et al.*, 1961; Komisaruk *et al.*, 1986; Kendrick *et al.*, 1988), cow (VanDemark and Hays, 1952) and the mare (Betteridge *et al.*, 1985; Aurich *et al.*, 1993; Sharp *et al.*, 1997; Vanderwall *et al.*, 1998). In our study, vaginal distension, cervical stimulation and intrauterine infusion of 500 ml PBS provoked a significant OT response. On several occasions shortly after PBS infusion the mares adopted a urinating posture or were actually expelling fluid,

often coinciding with OT peaks (data not shown). This observation is consistent with the findings of another study, where reflux of infused fluid often occurred within 10 to 15 minutes post-infusion (Jones, 1995) and it appears in our study that uterine distension provoked uterine contractions, probably elicited by OT release. In our mares, vaginal distension caused a significantly higher response than mating, oestrous and dioestrous teasing and uterine distension which were not statistically different from to each other. Although manipulation of the genital tract caused a significantly greater OT response than mating this is probably due to the longer period of time (12 min) that the stimuli were applied in comparison to the duration of mating in the mare (2 min; McDonnell, 1992). The results of our study demonstrated a gradation in the OT response depending on the duration and severity of the applied stimulus.

Peaks of OT were highest at the time of the stimulus application, as has been suggested earlier (Lehrer *et al.*, 1978), and declined rapidly reaching baseline levels by 30-60 minutes in all experiments. In agreement with the present study, jugular OT levels returned to baseline levels within 30 minutes after intravenous OT administration (Alexander *et al.*, 1995). Interestingly, where there was a measurable PGFM response, elevations tended to follow the OT peaks, indicating a possible temporal correlation between the two hormones as already suggested in sheep (Flint *et al.*, 1975; Roberts *et al.*, 1976; Sheldrick and Flint, 1985), cow (Mirando *et al.*, 1993; Fuchs *et al.*, 1996), women (Fuchs *et al.*, 1981b), rabbits (Small *et al.*, 1978) guinea-pigs (Leaver and Seawright, 1982) and mares (Vanderwall *et al.*, 1998). No PGFM response was detected at 2 of 5 mating and 3 of 5 teasing episodes. Also there was no prostaglandin release 16 to 18 hours after mating, although increased myometrial activity has been reported at that period of time after intrauterine infusion of bacteria (Troedsson *et al.*, 1993b). However, increased uterine activity could be due to local prostaglandins, released from the inflamed endometrium (Watson, 1989) and detected in uterine flushings (Watson *et al.*, 1987b).

Betteridge and coworkers (1985) showed in dioestrous mares that vaginal distension, uterine manipulation and cervical dilatation increased circulating PGFM levels in 50% of the mares and that rectal palpation and washing of the vulva did not affect PGFM concentrations. In our study, only one mare had a significant PGFM response to the manipulation of the genital tract. It has previously been observed that individual mares appear to have different capacities to release $PGF2\alpha$ in response to stimuli applied to the genital tract (Kask *et al.*, 1997). Furthermore, Goff and coworkers (1987) reported that during oestrus there was a dramatic decline in the PGFM response to exogenous OT possibly as a result of the low endometrial oxytocin receptor levels at this stage of the cycle (Sharp *et al.*, 1997). It also appears that the endometrium may require a period of exposure to progesterone before it has the capacity to release large quantities of $PGF2\alpha$ (Vernon *et al.*, 1981; Eggleton *et al.*, 1990). This may account for failure of $PGF2\alpha$ release in a number of oestrous mares and the low level of release in responding mares in comparison with concentrations measured at luteolysis (Goff *et al.*, 1987).

In conclusion, it was shown that teasing in oestrus and dioestrus caused OT release but the different behavioural responses indicate an effect of the steroid environment on responses to somatosensory stimuli mediated by OT in the brain. Both natural service and various stimuli applied to the genital tract, in many instances caused an increase in OT concentrations in the oestrous mare but incidence of $PGF2\alpha$ release was much lower. The effects of these ecbolic hormones may be important both peripherally, by enhancing gamete transport and evacuation of uterine contents after mating and centrally, in initiating sexual behaviour.

It could be that impaired uterine clearance in mares susceptible to PMIE is connected with differences in the ecbolic hormonal release patterns. Unfortunately, due to therapeutic considerations, mares susceptible to PMIE, which were mainly referred clinical cases, could not be used to compare ecbolic hormone release around natural service, since the introduction of semen into their uterus causes a massive infection. However, the use of artificial insemination has been encouraged in mares susceptible to

PMIE (Kenney *et al.*, 1975). In the next chapter artificial insemination will be used as a uniform stimulus to compare the patterns of ecbolic hormone release between mares resistant and susceptible to PMIE.

Chapter 5

Oxytocin and $PGF_{2\alpha}$ release in mares resistant and susceptible to persistent mating-induced endometritis

Introduction

The introduction of bacteria and sperm into the uterus at the time of breeding in the mare causes a transient inflammation of the endometrium (Kotilainen *et al.*, 1994). Uterine contractility promotes uterine clearance, eliminating cellular debris and uterine fluid (LeBlanc *et al.*, 1994b) presumably by drainage via the cervix and lymphatics. It has been suggested that mares failing to evacuate uterine fluid and cellular debris within 12 to 48 hours (LeBlanc *et al.*, 1994b; Katila, 1995) from the time of uterine challenge have impaired uterine contractility (Troedsson and Liu, 1991). These mares have been classified as having persistent mating-induced endometritis (PMIE). Mares susceptible to PMIE have reduced myoelectrical activity after the introduction of infection into the uterus compared with resistant mares (Troedsson *et al.*, 1993b).

Artificial insemination (AI) is widely used as a management tool to minimise uterine contamination in mares susceptible to endometritis (Kenney *et al.*, 1975). In other species the release of ecbolic hormones at the time of breeding (Fox and Knaggs, 1969; McNeilly and Ducker, 1972; Fuchs *et al.*, 1981a; Schams *et al.*, 1982; Todd and Lightman, 1986; Claus and Schams, 1990) directly influences uterine contractility and possibly assists with gamete transport (McNeilly and Folley, 1970; Wathes, 1984). Oxytocin can affect uterine motility both directly, and indirectly via the release of $PGF2\alpha$ (Poyser, 1995). Responsiveness of the uterus to OT and production and release of $PGF2\alpha$ is modulated via uterine OT receptors in the mare (Sharp *et al.*, 1997). It is possible that OT and $PGF2\alpha$ released at the time of AI in the mare could promote uterine clearance and that mares with PMIE could have deficient ecbolic hormone release.

The purpose of this study was firstly to investigate whether there were any differences in the release of OT and $PGF2\alpha$ before, during, and after AI with chilled extended semen

in mares resistant and susceptible to PMIE and secondly, to determine the responsiveness of the uterus of these mares to exogenous OT.

Materials and methods

Animals

Eleven fertile mares, aged 5 to 15 years, weighing 340 to 520 kg, classed as genitally normal, according to their reproductive history and endometrial biopsy scores of 1-2A (Kenney *et al.*, 1986), were used. The susceptible group of mares consisted of 13 mares, aged 7 to 18 years, weighing 370 to 620 kg, referred to our reproduction clinic with a history of subfertility related to uterine fluid accumulation for at least 48 hours after breeding. Only mares with no obvious conformational vulvar and perineal problems or cervical defects were included. Oestrus was detected by teasing with a stallion, combined with transrectal ultrasonographic examination of the genital tract. When the mare responded positively to teasing and uterine oedema was present, with a follicle of at least 35 mm present on the ovaries, the mare was considered to be in oestrus.

Experiment 1

Seven resistant and nine susceptible mares were used. On the day the mares were detected in oestrus, an indwelling cannula (13 gauge, Presidio Medico, Ecouen, France) was placed in the jugular vein under local anaesthesia after surgical preparation of the area. Blood samples for OT and PGFM, the main $PGF_{2\alpha}$ blood plasma metabolite (Goff *et al.*, 1984), were collected for 30 min, at 2 minutes intervals after which the mares were artificially inseminated following the hygienic procedures described by Watson (1995). Blood sampling continued at 2 minutes intervals during, and for 1 hour

after AI, and then for another hour, at 5 minutes intervals. Additional samples for PGFM were collected at 15 minutes intervals between 16 and 18 hours after AI.

Experiment 2

Resistant (n=11) and susceptible (n=10) oestrous mares had an indwelling cannula (13 gauge, Presidio Medico, Ecouen, France) placed in the jugular vein under local anaesthesia after surgical preparation of the area and blood samples were obtained at 5 minutes intervals, from 10 minutes before until 60 minutes after the intravenous administration of OT (1iu $20kg^{-1}$; Oxytocin-S, Intervet, Cambridge, UK).

Sample Handling

See Chapter 2.

Semen Collection

See Chapter 2.

OT assay

See Chapter 2.

PGFM assay

See Chapter 2.

Statistical Analysis

Baseline hormone concentrations were calculated from the mean of the values obtained prior to AI. Response to AI, for both hormones, was obtained from the mean values of the samples corresponding to the concentrations of the peak immediately after the application of the stimulus until hormonal concentrations returned to baseline levels (Table 5.1). Mean hormone concentrations were then calculated for every 30 minutes interval thereafter. Mean concentrations for both hormones at all time intervals and magnitude of response to AI, between the two groups, were compared using two sample t-tests.

Because of the pulsatile nature of OT release and its short half-life, responses were also assessed for individual mares. A mare was considered to have a positive OT response when the mean concentration of the peak immediately after the application of AI exceeded the mean baseline concentrations + 2 x SD. Mean PGFM concentrations were calculated for each mare as above (30 minutes intervals) and a positive response was recorded when the increase for each mare exceeded the mean baseline concentrations + 2 x intraassay coefficient of variation, in the first 30 minutes after AI. Numbers of mares from each group responding to AI with hormone release were compared using Fisher's exact test. In experiment 2, PGFM concentrations for both groups of mares were compared using a two sample t-test, for each 5 minutes intervals.

Results

Experiment 1

Baseline OT levels were not significantly different between the resistant and susceptible group (Table 5.1). There was a significant increase in OT concentrations in response to AI ($P<0.05$) in both groups when compared to baseline levels (Table 5.1). The mean

time from the start of the insemination procedure until OT concentrations returned to baseline was 7.24±0.92 minutes. There was no difference in the magnitude of the OT response to AI and also in the mean OT concentrations for any of the 30 minutes periods following AI between the two groups (Figure 5.1).

Table 5.1. Mean OT and PGFM concentrations for every 30 minutes before and after AI for resistant and susceptible mares.

	Resistant (n=7)		Susceptible (n=9)	
	Mean (±SEM) Hormone Concentrations (pg/ml)			
Time min	OT	PGFM	OT	PGFM
-30-AI	5.7±1.1	22.7±.0.7	6.3±1.9	20±0.0
AI	15.1±3.2	27.4±4.2	10.9±2.1	20±0.0
AI-30	7.6±2.3	34.1±6.0	8.5±2.2	20±0.0
30-60	9.6±2.8	47.8±9.5	8.4±1.9	20±0.0
60-90	8.4±2.3	139.2±95.8	6.7±1.7	20±0.0
16-18 h		32.6±11.5		45.2±18

There was also no significant difference between number of resistant (n=5; 71%) and susceptible (n=3; 33%) mares releasing OT in response to AI. PGFM concentrations did not increase at the time of AI in either group of mares, but mean PGFM levels for the first 30 minutes after AI were significantly higher ($P<0.05$) in the resistant group (Table 4.1). Furthermore, significantly more ($P<0.05$) resistant (n=5, 71%) than susceptible (n=0) mares released $PGF_{2\alpha}$ in response to AI (Figure 4.2).

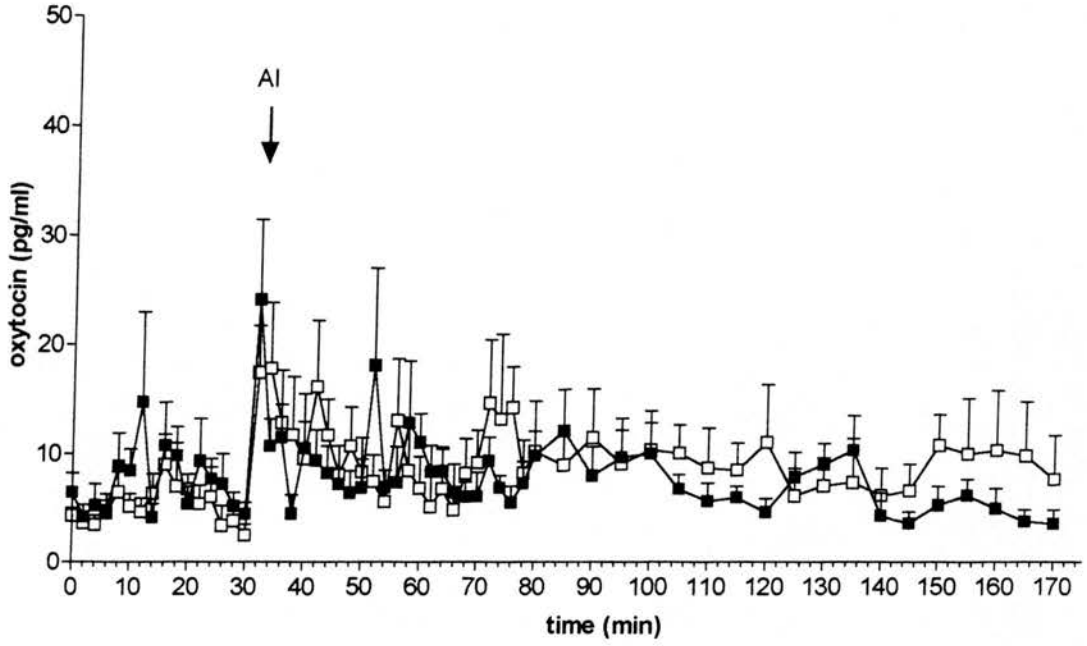

Figure 5.1. Oxytocin levels around artificial insemination in 7 resistant (□) and 9 susceptible (■) mares.

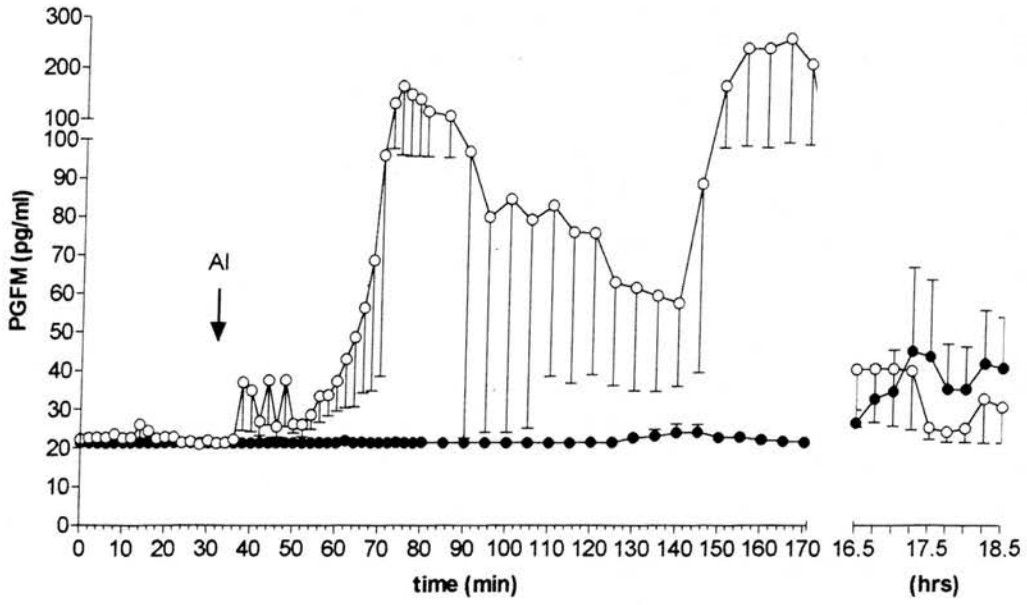

Figure 5.2. PGFM levels around artificial insemination in 7 resistant (○) and 9 susceptible (●) mares.

There was no significant difference, for both OT and PGFM, at any other 30 minutes period, between the resistant and susceptible group of mares, despite the very high PGFM concentrations in some of the resistant mares. There was no difference in mean PGFM concentrations 16 to 18 hours after AI between the two groups of mares and concentrations were not significantly higher than baseline samples.

Experiment 2

There was no difference in the mean baseline PGFM concentrations between the two groups prior to OT injection (Figure 5.3). The proportion of mares that released $PGF2\alpha$ in response to OT in the resistant group (n=10, 91%) was significantly higher ($P<0.05$) than in the susceptible group (n=4, 40%).

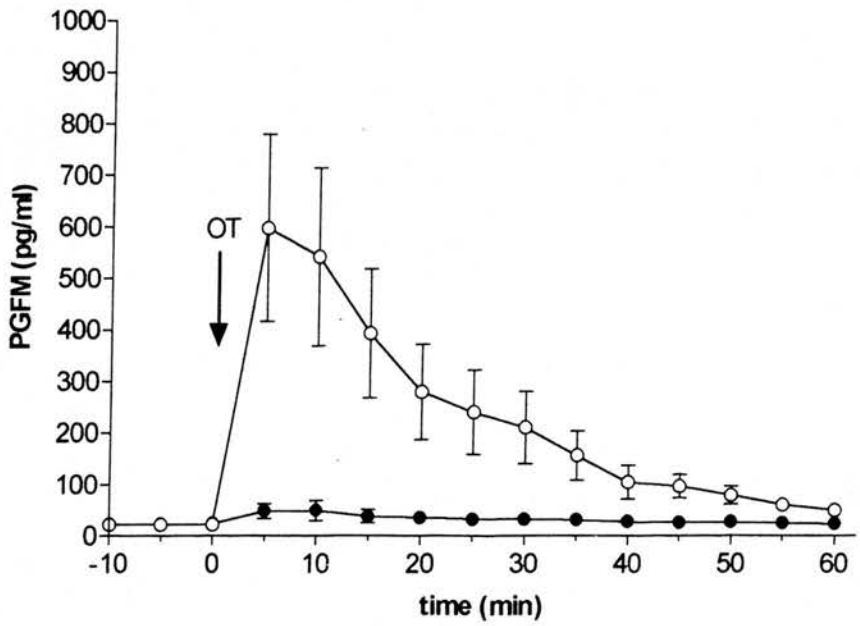

Figure 5.3. PGFM response after oxytocin injection in 11 resistant (○) and 10 susceptible (●) mares.

The PGFM concentrations of the resistant group were significantly higher ($P<0.05$) than the concentrations in the susceptible group at all times after the administration of OT until the end of the experiment (Figure 5.3). PGFM concentrations returned to baseline levels within 20 minutes for the responding susceptible mares but had not yet returned to baseline levels for the resistant group by the end of the experiment, at 60 minutes.

Discussion

The ecbolic hormones, OT and $PGF_{2\alpha}$ regulate myometrial contractility in many species (Seitchik and Chatkoff, 1976; Garcia-Villar *et al.*, 1983; Wheaton and Barbee, 1993; Carnahan *et al.*, 1996) including the mare (Goddard and Allen, 1985; Cross and Ginther, 1987; Ko *et al.*, 1989). Mares susceptible to endometritis have delayed uterine clearance and it has been suggested that they have impaired uterine contractility (Evans *et al.*, 1986; Troedsson and Liu, 1991; LeBlanc *et al.*, 1994b). This is the first study, in the current literature, which compares ecbolic hormone release profiles at the time of breeding in mares resistant and susceptible to endometritis.

Patterns of OT release have been reported in many species, however reports in the mare are contradictory and inconclusive (Burns *et al.*, 1981; Tetzke *et al.*, 1987; Stevenson *et al.*, 1991; Alexander *et al.*, 1995). Unlike ruminants (for review see Wathes, 1989), OT in the mare is released only from the posterior pituitary and not from the ovaries (Stevenson *et al.*, 1991) although a contribution from the uterus cannot be ruled out (Behrendt *et al.*, 1997; Watson *et al.*, 1997). Oxytocin binds to uterine receptors that are located in the endometrium and myometrium of the mare (Stull and Evans, 1986; Sharp *et al.*, 1994) and provokes uterine contractions by increasing the influx of calcium into the myometrial cells (Csapo, 1962). However, OT also stimulates uterine contractility

indirectly by mobilising arachidonic acid and initiating the production of uterine $PGF2\alpha$ (Poyser, 1995).

In this chapter, in agreement with Alexander et al. (1995) it has been shown that AI can stimulate the release of OT in mares. This is probably due to release occurring from the distension of the vaginal walls (Aurich *et al.*, 1993) and the manipulation of the cervix (Sharp *et al.*, 1994) at the time of the semen deposition into the uterus. However, other factors might play a role in the release of OT, including the distension of the uterine walls from the inseminate and the antigenic challenge of the sperm (see chapter 5). In the present study, OT concentrations before and after AI were similar in resistant and susceptible mares. Troedsson *et al.* (1993b) reported an initial increase in myoelectrical activity in both resistant and susceptible mares for the first 10 to 15 minutes after infusion of bacteria into the uterus which correlates well with the pattern of OT release observed after AI in this study. However there is no information on the relationship between plasma concentrations of OT and myometrial activity in the mare. In one study in the ewe it has been shown that minute doses of OT similar to endogenous pulses will stimulate uterine motility (Gilbert *et al.*, 1991) but this finding was not substantiated in another study (Garcia-Villar *et al.*, 1983). In the mare the low OT receptor content of the mare uterus at oestrus (Sharp *et al.*, 1997) does not correlate with the increased contractile response measured at this stage of the cycle (Jones *et al.*, 1991).

In the present study, mean PGFM concentrations were significantly elevated for the first 30 minutes after AI in the genitally-normal mares. Other studies have similarly shown release of $PGF2\alpha$ after manipulation of the tract at embryo transfer and after uterine lavage (Betteridge *et al.*, 1985; Watson *et al.*, 1988b; Kask *et al.*, 1997). In this study, the release of $PGF2\alpha$ might have been stimulated by the OT peak following AI indicating a temporal relationship between the two hormones as has previously been suggested in the mare (Sharp *et al.*, 1994; Vanderwall *et al.*, 1998). However an

important finding in this study was that no detectable concentrations of PGFM were measured in susceptible mares, despite the similarity of OT profiles between the two groups. There is little information on the relative importance of OT and prostaglandin in regulation of uterine contractions, however both appear to be involved in women (Fuchs, 1987) and rats (Maggi *et al.*, 1991). In reproductively normal mares, treatment with phenylbutazone, an inhibitor of prostaglandin synthesis at oestrus, has substantially interfered with uterine clearance (Cadario *et al.*, 1995) clearly indicating that endogenous prostaglandin is important in myometrial contractility. It is interesting to note that some mares which were classified as resistant had only low release of $PGF2\alpha$ at AI. The variability in ecbolic hormone response among mares, along with clinical observations (E.D. Watson, unpublished data) suggests that resistance and susceptibility to PMIE may include a spectrum of intermediate categories.

Susceptible mares have been described to have approximately a 2 hours delay in the onset of myometrial activity after intrauterine bacterial infusion and low myoelectrical activity from 11 hours after infection when compared to the high activity seen in resistant mares (Troedsson *et al.*, 1993b). In the period immediately after AI, prostaglandin release was low in susceptible mares which might explain the delay in myometrial activity reported earlier (Troedsson *et al.*, 1993b). However, in the present study there was no difference in the PGFM concentrations between resistant and susceptible mares 16 to 18 hours after AI. Troedsson *et al.* (1993b) suggested that the high myometrial activity in resistant mares was caused by prostaglandins released from activated inflammatory cells. It could be that the high prostaglandin concentrations produced by the inflamed endometrium (Watson, 1989) act locally and cannot be measured in the blood. However, in contrast to this, in a previous study elevated PGFM concentrations in mares with acute endometritis correlated well with uterine luminal $PGF2\alpha$ (Watson *et al.*, 1987b). In the present study therefore, there was no apparent

difference in circulating prostaglandin concentrations at 16 to 18 hours which could account for the difference in myometrial activity.

The significantly lower $PGF2\alpha$ release in the susceptible group of mares at the time of AI , despite the release of OT, suggested that there was a defect in $PGF2\alpha$ production. Administration of OT is used as an *in vivo* stimulus for $PGF2\alpha$ release in many species (Beard *et al.*, 1994; Mann and Lamming, 1995; Carnahan *et al.*, 1996), including the mare (Goff *et al.*, 1987) and acts via the OT receptor (Sharp *et al.*, 1997). In experiment 2, the significant response of the resistant mares to OT in oestrus was in marked contrast to Goff et al. (1987) who reported a dramatic decline in the $PGF2\alpha$ response of oestrous mares in response to OT administration. This may be due to the higher OT dose used in the present study. The ability to release prostaglandin in response to OT may explain the high contractile activity of the uterus at oestrus. More importantly it was shown that most susceptible mares failed to respond to OT and those that did, had a significantly lower response than the resistant group. This result may therefore explain the reduced prostaglandin response of the susceptible mares at AI and the reduced contractility of the uterus.

The administration of OT after breeding in mares is used in most of the treatment protocols for PMIE (Allen, 1991; LeBlanc, 1994; Pycock and Newcombe, 1996a), for the elimination of intrauterine fluid accumulations by provoking powerful contractions visible by transrectal ultrasonography (see chapter 7). However, even though OT administration has been shown to affect uterine activity in the susceptible mare (LeBlanc *et al.*, 1994b; see chapter 7) and can cause elimination of small volumes of fluid after inhibition of $PGF2\alpha$ synthesis in normal mares (Cadario *et al.*, 1995), some clinically affected mares fail to respond to OT therapy (E.D. Watson; unpublished data). In fact some of the mares included in this paper which totally failed to release $PGF2\alpha$ at AI, continued to accumulate fluid and failed to become pregnant, despite intensive flushing

and OT therapy. It may be that in these mares treatment with $PGF_{2\alpha}$ which has a sustained effect compared with OT (Troedsson *et al.*, 1995e; Combs *et al.*, 1996), could be a more appropriate form of therapy. However, the variability of prostaglandin responses after AI and OT administration amongst resistant and susceptible mares underlines that the separation of mares into two distinct categories may not always be appropriate.

It was concluded that OT release profiles do not differ, before, during and after AI between resistant and susceptible mares. However significantly fewer susceptible mares, when compared to resistant mares, released $PGF_{2\alpha}$ in response to endogenous or exogenous OT, indicating a defect at the receptor or post-receptor level. The variability in ecbolic hormone profiles suggested that there may be a gradation in susceptibility and resistance to PMIE in the mare.

In the past two chapters, the patterns of ecbolic hormone release were described around different reproductive stimuli, such as natural service and AI, in mares resistant and susceptible to PMIE. However, ecbolic hormone release is indicative of the activity of only the mechanical defence mechanism and not the uterine response at a cellular level. The introduction of sperm into the uterus, either during natural service or AI, is associated with an inflammatory reaction that should be resolved within 48h. In the next chapter, the cellular and bacteriological responses to these stimuli will be investigated.

Chapter 6

Does artificial insemination with chilled extended semen reduce the antigenic challenge to the mare's uterus compared with natural service?

Introduction

Susceptibility to uterine infection is the most important pathological cause of subfertility in mares. The effect of introduction of bacteria into the uterus of mares is well documented. Genitally-normal, so-called "resistant" mares will eliminate the infection within 48 to 72 hours (Adams *et al.*, 1987) whereas susceptible mares will remain persistently infected (Peterson *et al.*, 1969; Hughes and Loy, 1975). Thus, the use of artificial insemination (AI) in mares that are susceptible to infection is commonly recommended to reduce antigenic challenge to the uterus (Kenney *et al.*, 1975). However, there have been no reports comparing uterine response to AI versus natural service at a time when inflammation is resolving and the uterus is preparing to receive the embryo.

In the present study, uterine responses of resistant mares to AI and natural service were compared with that of susceptible mares to AI.

Materials and Methods

Animals

A total of 11 mares, 5 to 16 years of age, were used in this study. The mares were classified as either resistant or susceptible to endometritis based on their past reproductive history. Five mares (aged 5 to 1 2 year and weighing 350 to 500 kg) were classified as resistant based on the absence of uterine fluid, on <2% neutrophils present upon endometrial cytology and on endometrial biopsy categories I to IIA (Kenney *et al.*, 1986; Ball *et al.*, 1988). The remaining 6 mares (age 9 to 16 year and weighing 350 to 700 kg) were classified as susceptible from their reproductive history of subfertility and persistent infection after coitus. These mares had biopsy Categories of IIA to IIB. The

susceptible mares were free from uterine infection (based on uterine swabbings and absence of uterine fluid) prior to insemination. All II mares had good vulvar and perineal conformation and a relaxed cervix at oestrus.

On the day that the preovulatory follicle reached a diameter of 35 mm, as determined by transrectal ultrasonography, all of the mares received hCG (2,400 IU) intravenously. On the following day (Day 1),. they were inseminated artificially or were mated naturally. The mares were scanned again on Day 3 (48 hours after breeding) and by this time all had ovulated.

Semen from a fertile, 5-yr-old half-breed stallion free of genital pathogens was used for all inseminations and natural breedings. This stallion had achieved pregnancy rates in excess of 80% per oestrous cycle in the previous year.

Semen Collection

See Chapter 2.

Insemination

Resistant mares were artificially inseminated and bred by natural service during different oestrous cycles, whereas susceptible mares were client-owned animals whose owners would not permit their mares to be mated naturally. An interval of 26 to 62 d (mean = 41 d) elapsed between AI and natural service. The mares were prepared for AI and natural service in the same manner (Watson, 1995). Firstly, the tails were encased in plastic sleeves, then each mare's rectum was evacuated of faeces, and the perineal area and vulvar lips were washed 3 times with tamed iodine solution. The area was then dried with clean paper towels. Artificial insemination was performed using sterile equipment with a plastic surgeon's glove placed over a plastic sleeve on the operator's hand. Sterile K-Y jelly was used for lubrication.

The stallion's penis was washed with clean warm water and dried with clean paper towels prior to natural service.

Sampling Procedure

On Day 3 (48 hours after AI or natural service), the mares' ovaries and uteri were scanned by transrectal ultrasonography. The occurrence of ovulation and presence of uterine fluid was recorded. The volume of uterine fluid was assessed visually by comparison with ultrasonographic prints of known volumes infused into the uteri of dioestrous mares from chapter 3. The uterus of each mare was flushed as described in chapter 2.

Statistical Analysis

Numbers of cells and proportion of neutrophils recovered at flush after AI and natural mating were compared using a paired t-test. Differences in these measurements between resistant and susceptible mares were analysed using a two sample t-test. Tests on cell numbers were performed on log transformed data. Frequency of recovery of bacteria from mares after AI and natural service was compared using McNemar's exact test for paired data.

Results

Table 6.1 shows a summary of characteristics of uterine fluid recovered 48 hours after AI or natural service. Resistant mares rarely had appreciable quantities of uterine fluid after either AI or natural service. By contrast, all but two of the susceptible mares had moderate to large accumulations of fluid. Quality of recovered fluid did not differ between resistant mares after AI and natural service, and was never worse than only slightly cloudy. However, fluid from susceptible mares varied from cloudy to thick and

purulent. Cell numbers recovered from the fluid corresponded with visual inspection of the cloudiness of the fluid. There was no significant difference between AI and natural service in the resistant mares, but a much more intense inflammatory reaction occurred after AI in the susceptible mares ($P<0.001$).

Table 6.1. Analysis of fluid recovered from mares 48 hours after AI or natural service.

Mare	Volume of uterine fluid ml	Quality of recovered fluid	Cell number recovered $x10^4$/ml	Bacteria isolated	Percentage of Neutrophils
			Artificial Insemination		
Resistant					
1	-	crystal clear	0.2	-	72
2	-	slightly cloudy	21.3	-	76
3	-	clear	0.3	-	88
4	-	clear	16.3	-	81
5	<10	slightly cloudy	13.3	-	80
Susceptible					
1	>100	pus	153.3	>20 colonies	85
2	50-100	cloudy	346.7	>20 colonies	92
3	-	cloudy	686.7	-	79
4	>100	very cloudy	366.7	>20 colonies	89
5	-	cloudy	81.0	>20 colonies	90
6	>100	pus	152.0	-	63
			Natural Service		
Resistant					
1	<10	slightly cloudy	26.7	-	74
2	<10	slightly cloudy	5.0	<10 colonies	73
3	-	clear	8.0	N/D	72
4	-	slightly cloudy	11.3	>20 colonies	68
5	-	slightly cloudy	3.4	<10 colonies	76

The proportion of neutrophils was not different between AI and natural service in resistant mares or between resistant and susceptible mares after AI and was consistently high. Microorganisms were isolated from the uteri of 7 mares (4 susceptible and 3 resistant). The susceptible mares had yeast (n=1), mixed *Steptococcus zooepidemicus* and *Escherichia coli* (n=2) and *Enterobacter spp* (n=1). All of the infected resistant mares had *Streptococcus zooepidemicus*. Microorganisms were isolated more frequently (albeit in low numbers) from resistant mares after natural service than after AI although this did not reach significance ($0.05<P<0.1$).

Discussion

Uterine flushes collected from resistant mares prior to intrauterine challenge contain very low cell numbers and rarely contain neutrophils (Watson 1987b; Pycock and Allen, 1990). A significant uterine response to breeding was shown to be still present after 48 hours. Cytologic evaluation of cells in recovered uterine lavage fluid has been classified as diagnostic of endometritis if more than 2% of the cells are neutrophils (Ball *et al.*, 1988). Therefore, all of these mares had marked residual endometritis. In the resistant mares studied, by 48 hours there was no noticeable difference in the uterine response between AI, using commercially accepted procedures, and natural service despite low numbers of bacteria being isolated in flushes from some of the mares after natural service.

Neutrophil infiltration of the uterus peaks by about 6 hours after experimental induction of uterine inflammation by the introduction of high numbers of bacteria (Williamson *et al.*, 1987; Watson *et al.*, 1988b). The numbers remain elevated for at least 72 hours (Watson *et al.*, 1988b) and can remain elevated for more than 10 d (Williamson *et al.*, 1987). However, there is little information about uterine inflammation after breeding. The method of collection of cells influences the number recovered. Quantitative cytological and microbiological findings of low volume uterine lavage have proved to be

superior to those of endometrial swabbing (Ball *et al.*, 1988). A recent study which used intrauterine tampons to recover uterine secretions found that neutrophils had almost totally disappeared by 48 hours after AI (Katila, 1995). It seems likely that collection of cells by flushing procedure would provide a more representative profile of the cells present because of contact with a larger surface area of the endometrium and the avoidance of cells adhering to the cotton tampon.

In a previous study using genitally-normal mares in which fresh rather than chilled extended semen was used, there was no difference in the number of uterine neutrophils recovered 6 hours after AI or natural service (Kotilainen *et al.*, 1994). The presence of uterine neutrophils in resistant mares in the present study 48 hours after natural breeding and insemination should be noted in view of the common practice of reinseminating mares every 48 hours until ovulation. Leukocytes are known to phagocytose spermatozoa (Mattner, 1968) and to produce substances such as oxygen-free radicals which are cytotoxic (Waites and Bell, 1982). However, in the postpartum period, pregnancy rates in mares do not appear to affected by detection of neutrophils in endometrial or vestibular smears (Koskinen and Katila, 1987; Sertich and Watson, 1992), and studies in rabbits have shown that spermatozoa can pass through the genital tract and fertilize the ovum even in the presence of high numbers of neutrophils from an earlier mating (Taylor, 1982).

High numbers of neutrophils persisted in this study despite there being no growth of uterine pathogens from 6 of the flushings of the resistant mares after insemination or natural breeding and fewer than 10 colonies were isolated from 2 of the remaining flushes. Hinrichs *et al.* (1988) suggested that the growth of fewer than 10 colonies from uterine swabs represents contaminants or transient organisms. Kotilainen *et al.* (1994) demonstrated that either no bacteria or only very low numbers were recovered 6 hours after insemination. The present study confirms the conclusions of Kotilainen *et al.* (1994) that the intensity of the neutrophil reaction is dependent on the presence of spermatozoa and further establishes that bacterial populations do not increase after the 6

hours sampling. A recent *in vitro* study has shown that equine spermatozoa, but not seminal plasma are chemotactic for neutrophils (Troedsson *et al.*, 1995d), confirming the involvement of sperm antigens in post-service uterine neutrophilia. However, bacterial contamination of the uterus is an inevitable consequence of breaching the mare's cervix, even when sterile substances or semen along with extender and antibiotic are infused, which also undoubtedly contribute to initial inflammation postinsemination. Indeed, even transrectal manipulation of the cervix and uterus causes transient neutrophilia (Williamson *et al.*, 1987).

Detection of intrauterine fluid by ultrasonography is associated with uterine inflammation (Adams *et al.*, 1987). None of the resistant mares in the present study had large accumulations of intrauterine fluid 48 hours after AI or natural breeding.. Three mares, however, had a thin line of fluid that was not associated with isolation of significant numbers of bacteria from the uterine fluid. Differences in volumes and total (in contrast to progressively motile) numbers of spermatozoa inseminated between AI (40 ml; mean = $2.5x10^9$ sperm) and natural service (mean ejaculate volume for this stallion = 83 ml; $7.7x10^9$ sperm) did not appear to influence uterine response. Probably the two to three-fold difference in volume and sperm numbers between natural service and AI was not sufficient to cause a significant difference in the inflammatory response.

In the present study, the intense neutrophilia and uterine fluid accumulation in susceptible mares after AI was similar to results of previous studies upon infusion of sterile substances into the uterus (Watson *et al.*, 1987a; LeBlanc *et al.*, 1994b). However, this work appears to be the first study reporting detailed uterine response to AI in susceptible mares. All but 2 of the susceptible mares accumulated ultrasonically detectable fluid by 48 hours post AI, and the recovered lavage fluid from all of these mares was cloudier than that in any of the resistant mares, reflecting the higher numbers of cells. Bacteria were not isolated from the lavage fluid from two of the mares despite the presence of high numbers of neutrophils. This may have been due to the presence of infective agents such as mycoplasmas or anaerobes which were not cultured using

aerobic culture procedures, or to elimination of infection by 48 hours albeit with persisting sterile inflammation, presumably due to the continued presence of antigenic material.

In resistant mares, AI did not offer any advantage when compared with natural service in terms of uterine inflammatory response. This was unexpected in view of the numbers of bacteria introduced directly into the uterus with semen at natural service (approximately 0.5 million/ml) (Simpson *et al.*, 1975) compared with the inseminate at AI (no aerobic micro-organisms were cultured from the extended semen in this chapter, as was previously reported by Kenney *et al.* (1975) and the presence of low numbers of bacteria in the uteri of some of the mares after natural service. Only very low numbers of anaerobes have occasionally been isolated from samples of extended semen (Hoyumpa *et al.*, 1992), and, therefore, it is unlikely that anaerobes contributed to the inflammatory response. Perhaps this finding emphasizes the importance of nonbacterial antigens in post-service uterine neutrophilia.

It is not clear from this study that AI offers real advantage over natural service to susceptible mares, especially since it has been shown that AI in susceptible mares resulted in severe inflammatory response and persistent endometritis. However, many different factors are operative in susceptible mares which makes a direct comparison of breedings between resistant and susceptible mares impossible. For example, uterine contractility appears to be impaired in some susceptible mares (Troedsson *et al.*, 1993b; LeBlanc *et al.*, 1994b; Pycock, 1994;). In addition, changes in lymphocyte subpopulations are present in the endometrium of susceptible mares (Watson and Thomson, 1995), which may imply that in some mares distinct immunological factors are present that may contribute to susceptibility to infection. However, bacteria, albeit in low numbers, were isolated more frequently from resistant mares after natural service than after AI. Therefore, it would seem that minimizing bacterial contamination through AI would be the wise course in susceptible mares along with other pre- and post- AI therapies.

Such post-insemination therapies usually involve single or repeated OT administrations to enhance UCA and promote uterine clearance together with uterine flushes and antibiotic treatment (Pycock and Newcombe, 1996a). In the next chapter, the effect of OT administration on UCA will be investigated as well as the validity of ultrasonography, a widely used tool for the monitoring of the oestrous cycle of the mare, as a technique for measuring UCA in oestrous and dioestrous mares.

Chapter 7

The effect of transrectal ultrasonography and oxytocin on uterine contractile activity in resistant and susceptible mares

Introduction

Transrectal ultrasonography has proved to be an indispensable tool in equine reproduction for monitoring the oestrous cycle, diagnosing early pregnancy and further defining and understanding uterine and ovarian pathology (McKinnon *et al.*, 1987). Ultrasonography assists in the evaluation of volume, quality and distribution of intrauterine fluid accumulations and the effect of therapy on the evacuation of fluid in mares susceptible to persistent mating-induced endometritis (PMIE). In these mares defective uterine contractile activity (UCA) has been blamed for persistence of fluid accumulations in the uterus (Troedsson *et al.*, 1993b; LeBlanc *et al.*, 1994a). Uterine contractile activity in the mare has been studied in the past employing techniques such as intrauterine pressure catheters (Capraro *et al.*, 1977; Goddard *et al.*, 1985; Ko *et al.*, 1989), scintigraphy (LeBlanc *et al.*, 1994a; LeBlanc *et al.*, 1994b; Cadario *et al.*, 1995) and electromyography (Taverne *et al.*, 1979b; Haluska *et al.*, 1987; Jones *et al.*, 1991; Troedsson *et al.*, 1993a). In women, transvaginal and transabdominal ultrasonography have proved to be reliable methods for the evaluation of UCA (Kunz *et al.*, 1996; Leyendecker *et al.*, 1996; Kunz and Leyendecker, 1997) and in the cow and mare it has successfully been used to study uterine contractions during the oestrous cycle and early pregnancy (Cross and Ginther, 1987; Cross and Ginther, 1988; Griffin and Ginther, 1990; Bonafos *et al.*, 1994; Gastal *et al.*, 1998).

Oxytocin (OT) is currently used in the treatment of PMIE (LeBlanc, 1994; Pycock and Newcombe, 1996a) for enhancing uterine contractions and promoting uterine clearance. Oxytocin can affect UCA both directly, and indirectly via the release of prostaglandin (PG) $F_{2\alpha}$ (Sharma and Fitzpatrick, 1974; Roberts *et al.*, 1976). Responsiveness of the uterus to OT and release of $PGF_{2\alpha}$ is modulated via uterine OT receptors that are found both in the endometrium and the myometrium of the mare (Stull and Evans, 1986). The concentrations of these receptors vary during the oestrous cycle (Stull and Evans, 1986;

Sharp *et al.*, 1997) and it has been shown that the uterine myoelectrical response to OT is influenced by the stage of cycle (Troedsson *et al.*, 1995e).

The aim of the present study was to assess the use of transrectal ultrasonography in the measurement of UCA and to monitor the effect of OT administration on UCA in oestrous and dioestrous mares and in mares susceptible to PMIE. Experiments were designed to determine: a) the effect of transrectal ultrasonographic monitoring on UCA and on the release of OT and $PGF2\alpha$, b) the *in vivo* OT degradation rate in oestrous and dioestrous mares, c) the effect of OT administration on UCA scores in mares resistant and susceptible to PMIE, d) the effect of OT administration on PGFM concentrations in oestrous and dioestrous mares, and e) the effect of repeated OT administration on UCA in oestrous mares.

Materials and Methods

Animals

Twelve fertile mares of mixed breeding, aged 5-15 years, weighing 370-520 kg, and classed as resistant to PMIE, according to their reproductive history of high fertility and ability to rapidly expel intrauterine fluid after mating, were used. The susceptible group of mares consisted of 10 mares, aged 7 to 18 years, weighing 370 to 620 kg referred to the reproduction clinic with a history of subfertility related to intrauterine fluid accumulation for at least 48 hours after breeding. Only mares with no obvious conformational vulvar and perineal problems or cervical defects were included. Oestrus was detected by teasing with a stallion, combined with transrectal ultrasonographic examination of the genital tract. When the mare responded positively to teasing and uterine oedema was present, with a follicle of at least 35 mm present on the ovaries, the mare was considered to be in oestrus. Ovulation was detected ultrasonographically by

the disappearance of the follicle and the presence of a corpus luteum on the ovary of the mare. Day of ovulation was designated as Day 0.

Experiment 1

The mares (n=5) had an indwelling cannula (13 gauge, Presidio Medico, Ecouen, France) placed in the jugular vein aseptically under local anaesthesia, when detected in oestrus. The mares were then transrectally scanned using an Aloka ultrasound scanner (SSD-500V, Japan) with a 5MHz linear-array transducer and UCA was recorded on videotape for 10 minutes. Oxytocin and PGFM concentrations were measured in blood samples collected at 1 minute intervals.

Experiment 2

In order to determine the duration of increased circulating OT levels after exogenous OT administration, the OT concentrations in oestrous and dioestrous mares were investigated. When the resistant mares (n=10) were detected in oestrus an indwelling cannula (13 gauge, Presidio Medico, Ecouen, France) was placed in the jugular vein. Blood samples for measurement of OT concentrations were collected at 5 minutes intervals for 10 minutes before the administration of OT (1iu $20kg^{-1}$), and at 1 minute intervals for the next 10 minutes. The same procedure was repeated on day 7 after ovulation (n=6).

Experiment 3

Uterine contractile activity was monitored ultrasonographically and recorded on videotape for 10 minutes in 9 resistant and 5 susceptible mares in oestrus and in 8

resistant and 6 susceptible mares in dioestrus (Day 7 after ovulation). Uterine contractile activity was also recorded on video for 3 minutes before and then for 10 minutes after the intravenous administration of OT (Oxytocin-S, Intervet, Cambridge, UK; 1iu $20kg^{-1}$) to 12 resistant and 10 susceptible mares in oestrus and to 11 resistant and 6 susceptible mares in dioestrus (Day 7).

Experiment 4

In a preliminary study, mares were treated with a low dose of OT (0.01iu $20kg^{-1}$; Oxytocin-S, Intervet, Cambridge, UK). As no PGFM response was measured with this dose, all subsequent mares received a higher dose. Resistant oestrous mares (n=11) had an indwelling cannula (13 gauge, Presidio Medico, Ecouen, France) placed in the jugular vein and blood samples were obtained at 5 minutes intervals, from 10 minutes before, until 60 minutes after the intravenous administration of OT (1iu $20kg^{-1}$). The same procedure was repeated on day 7 (n=7) after ovulation.

Experiment 5

Oestrous mares (n=4) had a cannula placed in the jugular vein and their uterus was scanned transrectally and videotaped for 5 minutes before and 10 minutes after the intravenous administration of OT (1iu $20kg^{-1}$). The same procedure was repeated 4 times at an interval of 2.5 hours.

Sample handling

See Chapter 2.

OT assay

See Chapter 2.

PGFM assay

See Chapter 2.

Videotape Analysis

See Chapter 2.

Statistical analysis

In experiment 1, the linear relationship between mean UCA scores and mean OT and PGFM concentrations was tested using regression analysis. In experiments 2 and 3, mean OT concentrations between oestrous and dioestrous mares and mean UCA scores of oestrous and dioestrous resistant and susceptible mares were compared using a two sample t-test. Mean baseline UCA scores prior to OT administration were compared to mean scores of the control groups using a paired t-test. The duration of the OT effect on UCA between resistant and susceptible oestrous mares in experiment 3 and the differences between mean baseline PGFM concentrations and the response to OT administration for each 5 minutes intervals in experiment 4, were compared using a two sample t-test. Mean PGFM concentrations prior to OT administration for oestrous and dioestrous mares were compared using a paired t-test. In experiment 5, mean baseline UCA scores between OT injections were compared using a one-way ANOVA test. The effect of OT administration on UCA scores was compared to mean baseline values using a two sample t-test.

Results

Experiment 1

Mean OT, PGFM levels and mean UCA scores remained constant and did not change significantly at any time throughout the experiment. Mean OT concentrations were barely above the detection limit of the assay (0.8 pg/ml) and mean PGFM concentrations were also at low levels. There was no significant relationship between mean UCA scores and mean OT ($P>0.1$) and PGFM ($P<0.1$) concentrations (Figure 7.1).

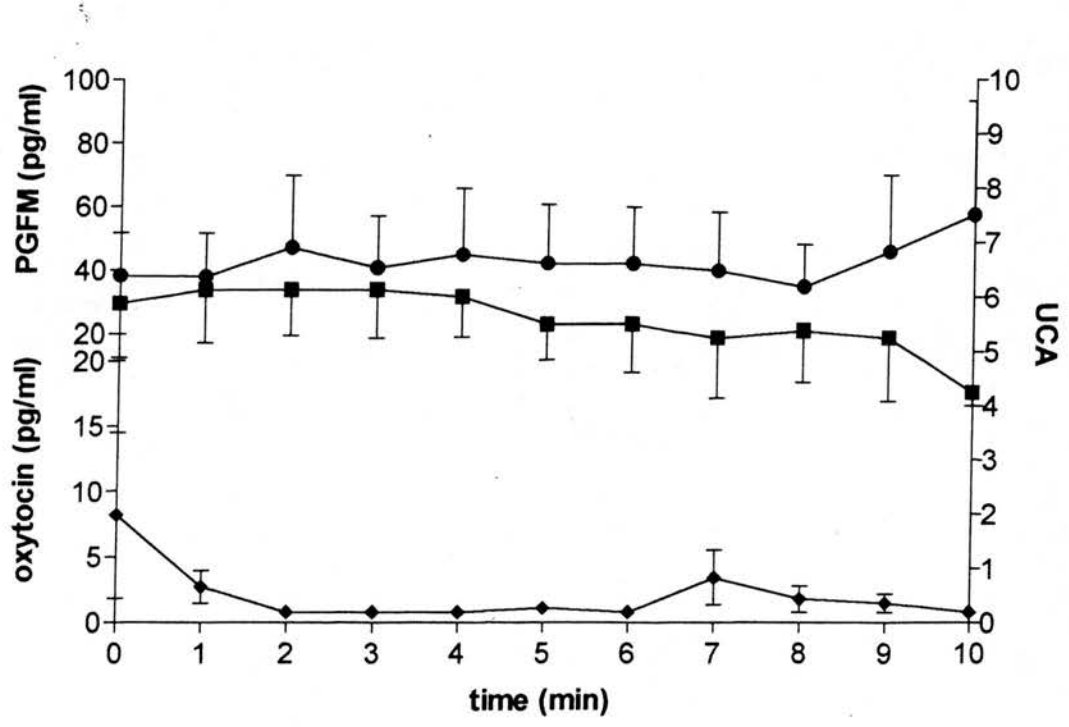

Figure 7.1. Uterine contractile activity (■), oxytocin (◆) and PGFM (●) concentrations during transrectal scanning.

Mean circulating OT concentrations after administration of OT were significantly higher ($P<0.001$) than mean baseline values at all times in both groups of mares (Figure 7.2). Values peaked in oestrus and in dioestrus, in the second and first minute respectively, after OT administration. There was no statistically significant difference between oestrus and dioestrus in OT concentrations at any of the sampling times. Oxytocin levels in both groups remained significantly higher ($P<0.05$) than mean baseline concentrations until the end of the experiment. However, in oestrus, concentrations had decreased by minute 9 ($P=0.06$) and minute 10 ($P<0.01$).

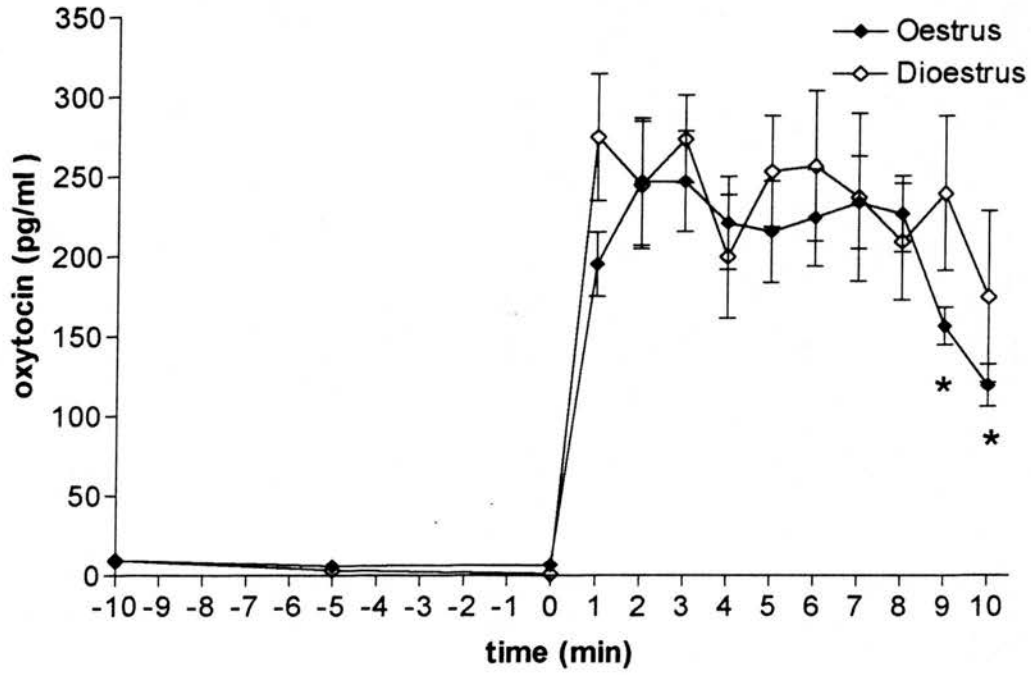

Figure 7.2. Oxytocin concentrations after OT administration in 4 oestrous (◆) and 4 dioestrous (◊) mares (* $P<0.05$ compared with peak response).

Uterine contractile activity was significantly higher ($P<0.001$) in the resistant oestrous mares than in all other groups of mares. There was no significant difference between mean UCA scores of resistant dioestrous or susceptible dioestrous and oestrous mares (Figure 7.3). In oestrus, the administration of OT caused a significant decrease ($P<0.05$) in UCA scores in both groups of mares (Figure 7.4). Uterine contractile activity scores returned to baseline levels significantly faster ($P<0.05$) in resistant (6 min) than in susceptible (8 min) mares after OT administration. In dioestrus, the administration of OT caused a decrease in UCA scores that did not reach statistical significance in either group of mares.

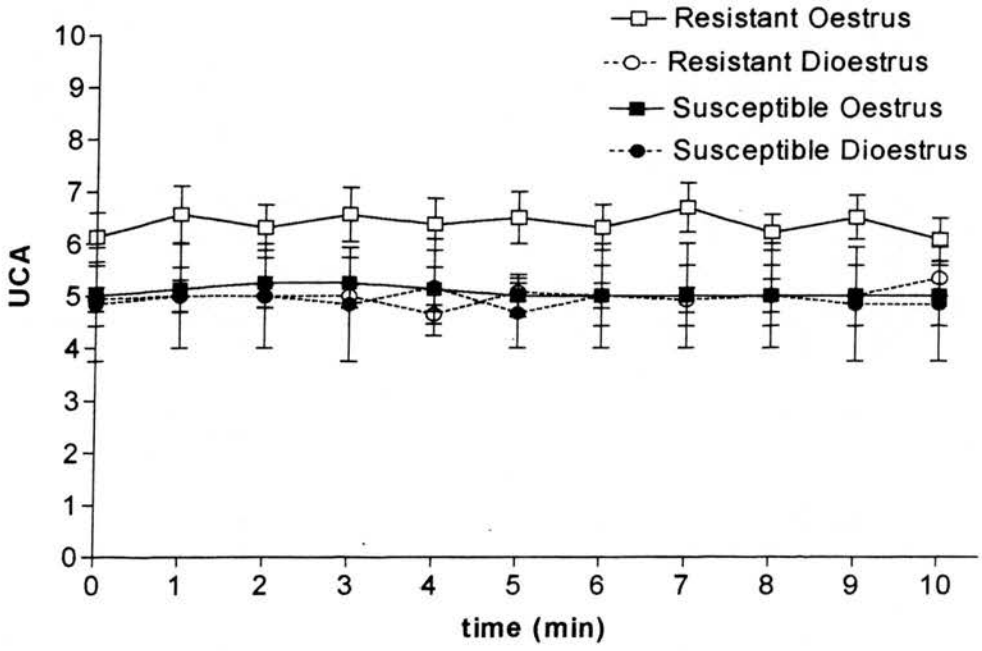

Figure 7.3. Uterine contractile activity in resistant and susceptible mares in oestrus and dioestrus.

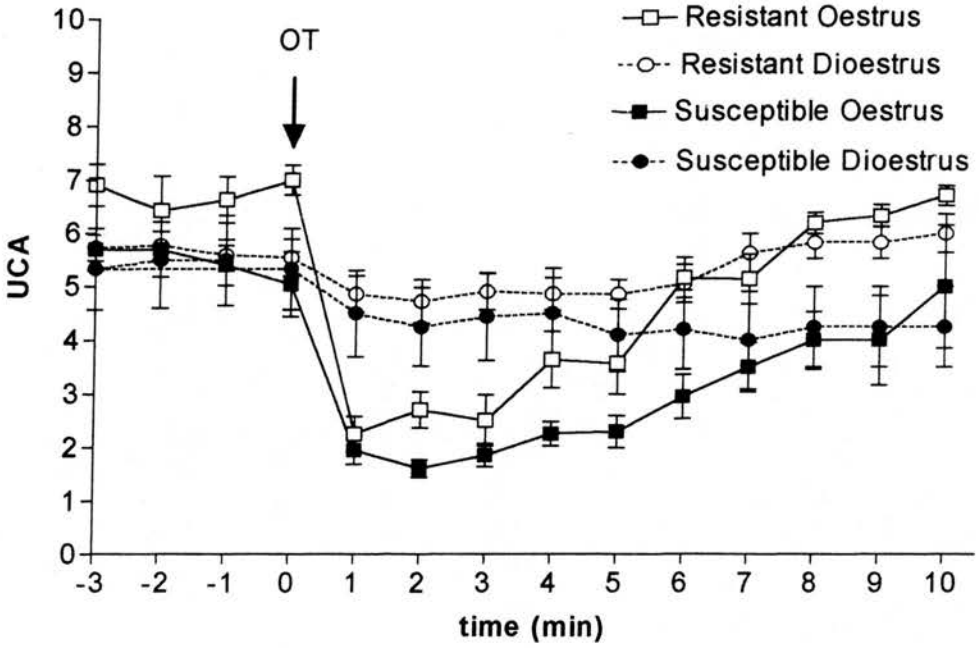

Figure 7.4. Uterine contractile activity before and after OT administration (1iu/20kg) to resistant and susceptible mares in oestrus and dioestrus.

Experiment 4

In oestrus, PGFM concentrations were significantly higher ($P<0.05$) than mean baseline values within 5 minutes from OT administration and remained significantly higher until the end of the sampling period (Figure 7.5). In dioestrous (day 7) mares, mean PGFM concentrations were significantly higher ($P<0.05$) than mean baseline values only for the first 10 minutes after the administration of OT. The PGFM response to OT was significantly greater ($P<0.05$) in oestrous than dioestrous mares.

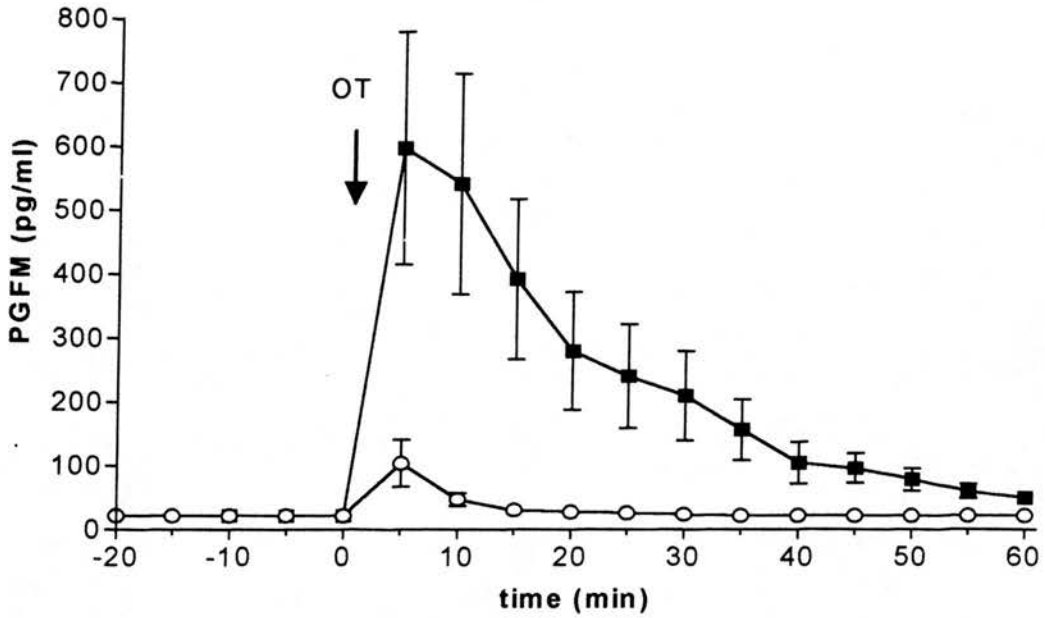

Figure 7.5. PGFM levels before and after OT administration (1iu/20kg) to 7 oestrous (■) and 6 dioestrous (○) mares.

Experiment 5

There was no significant difference in mean baseline UCA scores between the four periods prior to the administration of OT (Figure 7.6). The administration of the first OT injection caused uterine spasm and reduced UCA significantly ($P<0.05$) for the first 4 minutes.

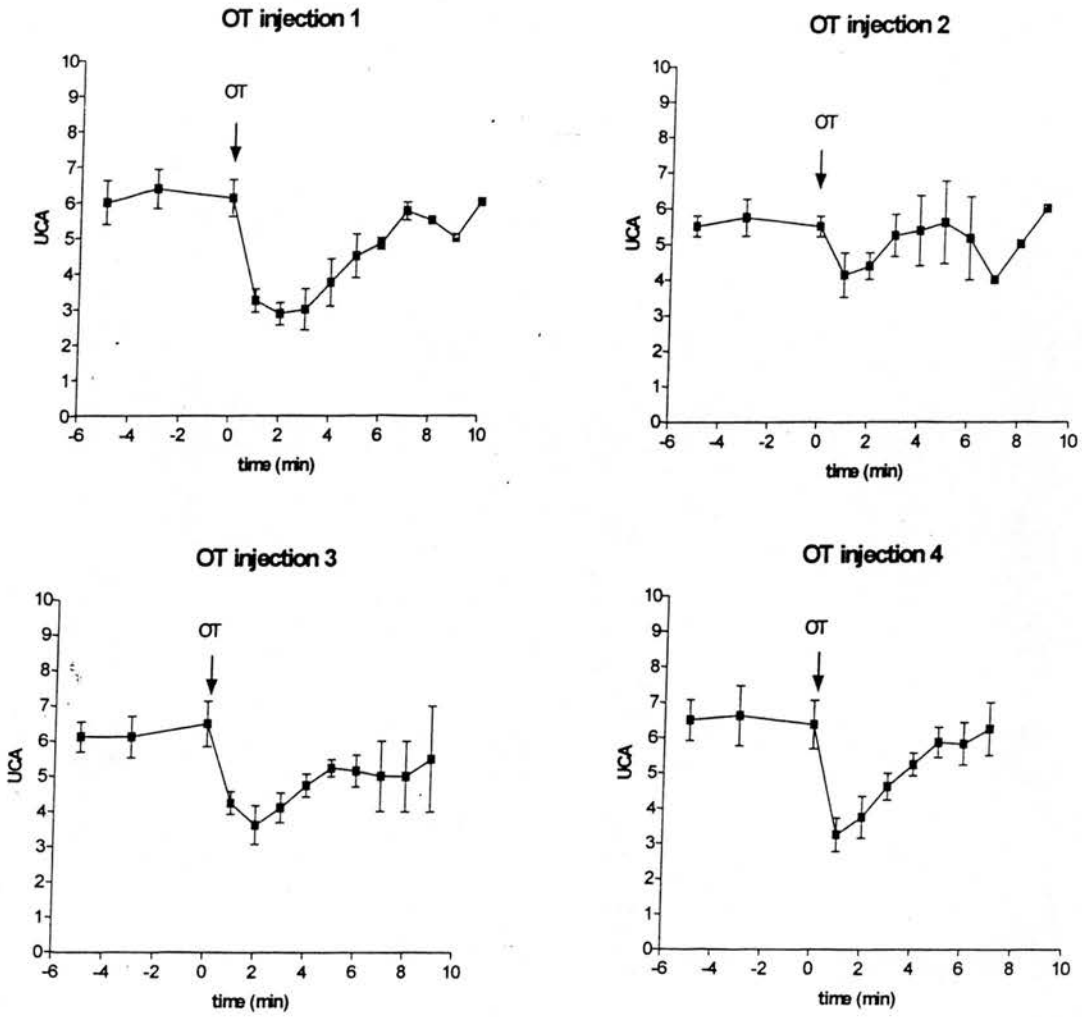

Figure 7.6. Uterine motility (■) after repeated OT injections in 4 oestrous mares.

Discussion

Ultrasonography is a technique available to all veterinary practitioners and is a relatively noninvasive means of assessing uterine contractility when compared to the introduction of intrauterine uterine pressure (IUP) catheters into the uterus, or surgical implantation of electrodes into the uterine parenchyma. It is also cheap when compared

with the expense of purchasing and maintaining scintigraphic equipment. Although ultrasonography has a stimulatory effect on uterine myoelectrical activity (M. Troedsson, personal communication) it was found that it did not have any influence on either visual UCA scores or ecbolic hormonal release. The low OT and PGFM concentrations reported in experiment 1 are in agreement with other studies (Tetzke *et al.*, 1987; Stevenson *et al.*, 1991; Sharp *et al.*, 1997). No "spurt" OT release was observed as a result of ultrasonography and PGFM levels remained at concentrations barely above the detection limit of the assay. It seems probable that some differences detected in myoelectrical activity may be too subtle to be visible on ultrasonography. However the lack of an OT and PGFM response to ultrasonography strongly suggests that this technique has very little effect on uterine function. Protracted or vigorous ultrasonographic examinations might however induce sufficient stress to alter UCA through changes in ecbolic hormone concentrations.

A strong correlation between OT release and UCA has recently been reported in the mare (Madill *et al.*, 1998). As OT is used as a therapy for enhancing uterine clearance in mares susceptible to PMIE (LeBlanc, 1994) it is important to know the *in vivo* kinetics of OT after injection and to relate this to the duration of uterine contractions. Concentrations of OT in the mare after administration of a pharmacological dose of OT, reported in experiment 2, are considerably higher than at other physiologic events, such as mating, teasing and artificial insemination, which elicit OT release as has been shown in chapter 3 (Nikolakopoulos *et al.*, 1998a). The relationship between the OT dose used in this study and the maximal OT values for both the oestrous and dioestrous mares and are very similar to the concentrations reported by Stevenson *et al.* (1991) in 2 mares at unspecified stages of the oestrous cycle. In another study by Shand *et al.* (1995) the administration of a similar OT dose to stallions resulted in OT concentrations a magnitude higher than the concentrations reported in this study. In both of these reports (Stevenson *et al.*, 1991; Shand *et al.*, 1995), as well as in oestrous mares in the present study, levels of circulating OT were significantly reduced by 10 minutes after OT administration. It could be that uterine OT receptors in the mare bind the OT molecules

and in this way reduce the amount of free circulating OT compared with the stallion. However, if that were the case, then OT concentrations should decrease rapidly after OT administration at the time of the observed uterine spasm. The consistently high OT concentrations in the peripheral circulation at the time of uterine spasm suggests that the uterus could be an alternative source of OT. In a recent report high levels of immunostaining for OT was reported in the oestrous endometrium (Watson *et al.*, 1998).

In dioestrus, high OT concentrations throughout experiment 2 correspond with the ultrasonographic findings of experiment 3, where OT administration did not affect UCA scores. Since concentration and affinity of uterine OT receptors do not change between oestrus and day 8 after ovulation (Sharp *et al.*, 1997) the effect of OT on UCA in dioestrus might be due to the rising progesterone concentrations (Plotka *et al.*, 1975; Townson *et al.*, 1989). Rising progesterone concentrations in early dioestrus increase electrical resistance between myometrial cells (Ichikawa and Bortoff, 1970) and therefore suspend the propagation of action potentials (Marshall, 1959) and induce asynchronous activity (Csapo 1961).

In the present study, resistant mares had significantly higher UCA scores in oestrus than on D7 of dioestrus. Although direction and speed of the uterine contractions were not evaluated in the present study, rapid changes in these parameters could clearly be seen in the uterine peristaltic motion of oestrous mares but not in dioestrous mares. Intense peristaltic motion was clearly visible in oestrus in the form of "waves" travelling on the longitudinal uterine axis from the fundus towards the cervix. However, on many occasions the direction of the peristaltic wave was reversed and/or its speed markedly altered. In dioestrus, uterine peristaltic motion was more uniform and individual "waves" were not as discernible as in oestrus. This may be explained by the work of Troedsson *et al.* (1993a) who reported that although uterine activity bursts are of longer duration in dioestrus, they occur more frequently and are of shorter duration in oestrous mares. Griffin and Ginther (1990) using ultrasonography reported a significant

decrease in the uterine contractility scores from the day of ovulation to day 1 and a subsequent increase between days 2 and 4. However in that study uterine contractility scores were not measured in oestrus.

Impaired UCA is thought to contribute to the inability of the susceptible uterus to clear intrauterine fluid accumulations (Troedsson *et al.*, 1993b; LeBlanc *et al.*, 1994a; see chapter 3), though other factors may be involved such as functional disturbances of the endometrium (Schoon *et al.*, 1998) or uterine positioning (LeBlanc *et al.*, 1998). In the present study it was shown that oestrous mares susceptible to post mating intrauterine fluid accumulations had significantly lower baseline UCA than genitally normal mares. This disagrees with another study which found no difference in frequency, duration and intensity of myoelectrical activity between resistant and susceptible mares prior to intrauterine infusion of bacteria (Troedsson *et al.*, 1993b). However in the same study susceptible mares did display asynchrony of uterine electrical activity between the implantation sites that failed to reach significance when compared to resistant mares. It could be that the asynchrony in UCA is perceived ultrasonographically as uncoordinated uterine activity and resulted in lower UCA scores.

In the present study, a significant reduction in UCA scores was visually observed within 1 minute from OT administration in both resistant and susceptible oestrous mares. This further confirms the ability of OT to enhance impaired UCA and increase uterine clearance, within 8 minutes from OT administration, in mares susceptible to PMIE as previously shown by LeBlanc (1994). Uterine spasm of the myometrium was seen after intravenous OT administration as a shortening of the vertical uterine axis combined with a visual decrease of UCA. Increased uterine activity was also observed immediately after OT administration in several studies utilizing electromyography (Jones *et al.*, 1991; Troedsson *et al.*, 1995e) and IUP transducers (Goddard and Allen, 1985; Ko *et al.*, 1989). In these latter studies uterine activity was reported to remain increased for 1 hour as detected by electromyography (Troedsson *et al.*, 1995e) and 10 to 30 minutes when measured with IUP transducers (Goddard and Allen, 1985; Ko *et al.*, 1989). However,

the majority of radiocolloid was cleared from the uterus within 15 minutes of OT administration (LeBlanc *et al.*, 1994b). Therefore it seems that the early contraction visible by ultrasonography is responsible for clearance of most of any intraluminal fluid. In addition, it was shown that OT concentrations are significantly decreased by 10 minutes after injection, confirming a relationship between OT concentrations and uterine activity as suggested by Madill *et al.* (1998).

The OT administration on day 7 of dioestrus failed to elicit statistically significant changes in UCA in both groups of mares in disagreement with the electromyographic data of Ko *et al.* (1989) and Troedsson *et al.* (1995e) who reported a similar intensity of uterine electrical activity after administration of OT in oestrous and dioestrous mares. The discrepancy with the results presented in this chapter could be due to the higher OT dose (40 USP) used (Ko *et al.*, 1989), and/or the stage of dioestrus in which the experiments were performed (Troedsson *et al.*, 1995e), which was not defined. It has been shown that the release of $PGF2\alpha$ in response to OT changes throughout dioestrus (Goff *et al.*, 1987). In that study there were significant differences in the release of $PGF2\alpha$ between various days, with the release being low in mid-dioestrus. Elevated $PGF2\alpha$ concentrations will stimulate UCA and consequently uterine clearance (Combs *et al.*, 1996). In this study OT-stimulated release of $PGF2\alpha$ was very low on day 7 which would suggest that the effect of OT on UCA might be reduced at this time in dioestrus. By contrast, OT elicited a very high release of prostaglandin during oestrus in association with the greater UCA which was recorded at this time. In the present study a high PGFM response to OT administration in oestrus was shown that is in marked contrast to Goff *et al.* (1987) who reported a dramatic decline in the $PGF2\alpha$ response of oestrous mares in response to OT administration. This could be due to the different OT doses used and/or stage of oestrus.

The elimination of intrauterine fluid accumulations is based on the ability of the uterus to actively contract in order to promote cervical and lymphatic drainage. Therefore, it appears logical that the effectiveness of UCA is directly proportional to the number of

organized uterine contractions elicited by OT administration. The repeated administration of OT over 1 day is now commonly used as a method of enhancing uterine clearance in mares with PMIE. In the present study although the effect of the first OT injection on UCA was similar to experiment 2, the other OT injections had diminished effect. This agrees with findings in the ewe (Sheldrick and Flint, 1986) and the mare (Betteridge *et al.*, 1985) where repeated OT administrations caused a gradually diminished $PGF_{2\alpha}$ response suggesting that a period of uterine refractoriness follows OT administration. This finding suggests that the current use of repeated OT injections in susceptible mares may vary in efficacy depending on the interval between injections.

It was concluded that transrectal ultrasonography was a useful tool for monitoring and evaluating UCA and did not appear to stimulate ecbolic hormone release. Oestrous resistant mares had significantly higher spontaneous UCA than susceptible mares, as detected by ultrasonography. Both groups of mares responded to OT administration with uterine spasm, however the repeated use of OT at short time intervals caused uterine refractoriness in resistant mares and therefore may be less effective at clearing fluid than the first time it is administered. Stage of cycle also affected response to OT as measured by UCA. This could not be explained by differences in OT concentrations after injection. Differences in baseline UCA scores, reported in this study, in combination with other parameters, could function as a predictor of susceptibility to PMIE. Further studies are needed to determine the effect of different OT doses and regimes on uterine clearance.

The difference in the myometrial response to the OT administration at oestrus and at day 7 after ovulation, as detected by ultrasonography, is explained by the sharply increasing progesterone levels in the early postovulatory period. However, the precise days after ovulation in which OT can be effectively used is of great clinical and therapeutical importance. In the next chapter the effect of OT on UCA in the early postovulatory period will be investigated.

Chapter 8

Effect of oxytocin administration on uterine contractile activity and progesterone concentrations in the early postovulatory period in the mare

Introduction

The uterus responds to breeding with a transient inflammatory reaction (Katila, 1995; Katila, 1997) caused by the chemotactic effect of equine sperm (Kotilainen *et al.*, 1994; Troedsson *et al.*, 1995d; Nikolakopoulos and Watson, 1997b – see chapter 5) and the introduction of bacterial contamination at breeding (Peterson *et al.*, 1969; Hughes and Loy, 1975). The inflammatory products are expelled from the uterine lumen within 12 to 48 hours by uterine contractions that promote cervical and lymphatic clearance (Troedsson and Liu, 1991; LeBlanc *et al.*, 1994a; LeBlanc *et al.*, 1995c; Troedsson, 1997). Failure to eliminate intrauterine fluid and cellular debris after breeding results in the mare developing persistent mating-induced endometritis (PMIE) (Troedsson *et al.*, 1995a) and reduces the survival chances of the embryo after its descent into the uterus on about day 6 after ovulation.

Oxytocin (OT) is used in the treatment of PMIE to enhance uterine contractility and promote the elimination of intrauterine fluid after breeding (Allen, 1991; LeBlanc, 1994; Pycock and Newcombe, 1996a). Oxytocin causes contraction of the myometrium both directly and indirectly via endometrial $PGF_{2\alpha}$ release (Sharma and Fitzpatrick, 1974; Roberts *et al.*, 1976). The effect of OT on the myometrium is modulated by uterine OT receptors that are found in the endometrium and myometrium of the mare (Stull and Evans, 1986). The numbers of OT receptors are regulated by the levels of circulating ovarian steroids; lowest uterine OT receptor densities were observed at oestrus and day 8 after ovulation and the highest at day 14 of the cycle (Sharp *et al.*, 1997). In the mare, progesterone concentrations remain low during oestrus and increase rapidly within 12 hours after ovulation (Plotka *et al.*, 1975; Townson *et al.*, 1989) to reach a plateau by day 6. It is possible that the high circulating concentrations of progesterone after ovulation may affect the uterine response to OT in the early postovulatory period. In chapter 4 (Nikolakopoulos *et al.*, 1998b), it was shown that the administration of a

pharmacological dose of OT can result in high prostaglandin release in oestrous mares. However, Goff *et al.* (1987) reported only low levels of prostaglandin release after OT administration in early dioestrus.

The use of OT after mating promotes drainage of uterine fluid (LeBlanc *et al.*, 1994b; Pycock and Newcombe, 1996a) and is thought not to adversely influence gamete transport and function in the oviduct (Allen, 1991). When mares are bred naturally, mares can be mated early and postbreeding treatments can be performed in late oestrus (Pycock and Newcombe, 1996a). However, when artificially inseminating with chilled or frozen semen, highest pregnancy rates are achieved when insemination is performed around ovulation (Voss *et al.*, 1982; Vidament *et al.*, 1997). Therefore almost all treatments after artificial insemination are carried out in the early postovulatory period. Anecdotally, OT has been used at day 7 and 8 to aid in the expulsion of fluid at embryo flush (E.D. Watson, unpublished data); however there are no detailed data on the effect that OT has on uterine contractile activity (UCA) during the first five days after ovulation when post-breeding therapies can be carried out. There was no difference in the effect of OT on UCA between oestrus and days 1 and 8 after ovulation as measured by electromyography (Ko *et al.*, 1989) which suggested that in the mare oestrogen priming is not necessary for the uterus to respond to OT. In contrast LeBlanc and coworkers (1994a) showed that uterine clearance of radiocolloid was negligible on day 5 and 6 of dioestrus which might reflect the low release of OT-induced prostaglandin in early dioestrus (Goff *et al.*, 1987). Apart from limited data on the efficacy of OT in inducing uterine clearance during early dioestrus (LeBlanc *et al.*, 1994b), there is no information on the effect of daily injection of therapeutic doses of OT on the developing corpus luteum (CL). Neely *et al.* (1979) showed that daily administration of OT between days 4 and 8 failed to induce luteolysis. However no studies have measured the effect of OT on the function of the early developing CL.

Ultrasonography has been employed as a non-invasive technique for the direct observation of UCA in mares (Cross and Ginther, 1987) and cows (Bonafos *et al.*, 1994) and is currently used to monitor intensity and direction of uterine peristalsis in women (Lyons *et al.*, 1991; Kunz *et al.*, 1996; Leyendecker *et al.*, 1996). In the present study, ultrasonography was employed to evaluate the effect of OT on UCA in the early postovulatory period in the mare and also to measure plasma progesterone concentrations to monitor the effect of OT on CL function.

Materials and Methods

Animals

Five fertile mares, aged 5-15 years, weighing 370-510 kg, classed as resistant to PMIE, according to their reproductive history of not accumulating uterine fluid after breeding, high fertility, negative endometrial cytology and bacteriology, and endometrial biopsy scores of 1-2A (Kenney *et al.*, 1986), were used. Ovulation was detected ultrasonographically by the disappearance of the preovulatory follicle and presence of a corpus luteum on the ovary of the mare. The day of ovulation was designated as day 0.

Experimental Protocol

Transrectal ultrasonography was performed using an Aloka scanner (SSD-500V, Japan) with a 5MHz transducer. Uterine contractile activity was recorded on videotape for 1 minute before, and 7 minutes after the administration of a single intravenous injection of oxytocin (Intervet, Cambridge, UK, 1iu/20kg bodyweight) from the day of ovulation until day 7 after ovulation. A blood sample was collected by jugular venipuncture into heparinised evacuated tubes, once daily prior to OT administration. The same procedure was repeated for 6 minutes in another cycle without the administration of OT. Blood

samples were immediately centrifuged at 2000 *g* for 15 minutes at 4°C and the plasma was stored at -20°C until assayed for progesterone.

Videotape Analysis

See Chapter 2.

Progesterone Assay

See Chapter 2.

Statistical Analysis

Mean daily progesterone concentrations between cycles were compared using a paired t-test. Uterine contractile activity before and after the administration of OT from day 1 to 7 was analysed using a one sample t-test and responses were compared between days using a one-way ANOVA. Uterine activity in the treatment and the control cycles was compared using a paired t-test. Differences were considered to be significant when $p<0.05$.

Results

Progesterone concentrations were already elevated on Day 1 after ovulation and gradually increased up to Day 7. There was no statistical difference between progesterone concentrations in the treatment and control cycles (Figure 8.2).

Ultrasonographic scanning did not affect UCA scores supporting the findings of chapter 3. There was no significant difference between mean baseline UCA scores for each day in the control and the OT-treatment cycle. In response to OT administration, UCA scores significantly decreased ($P<0.05$) within 1 minute up to day 3 postovulation (Fig. 8.1).

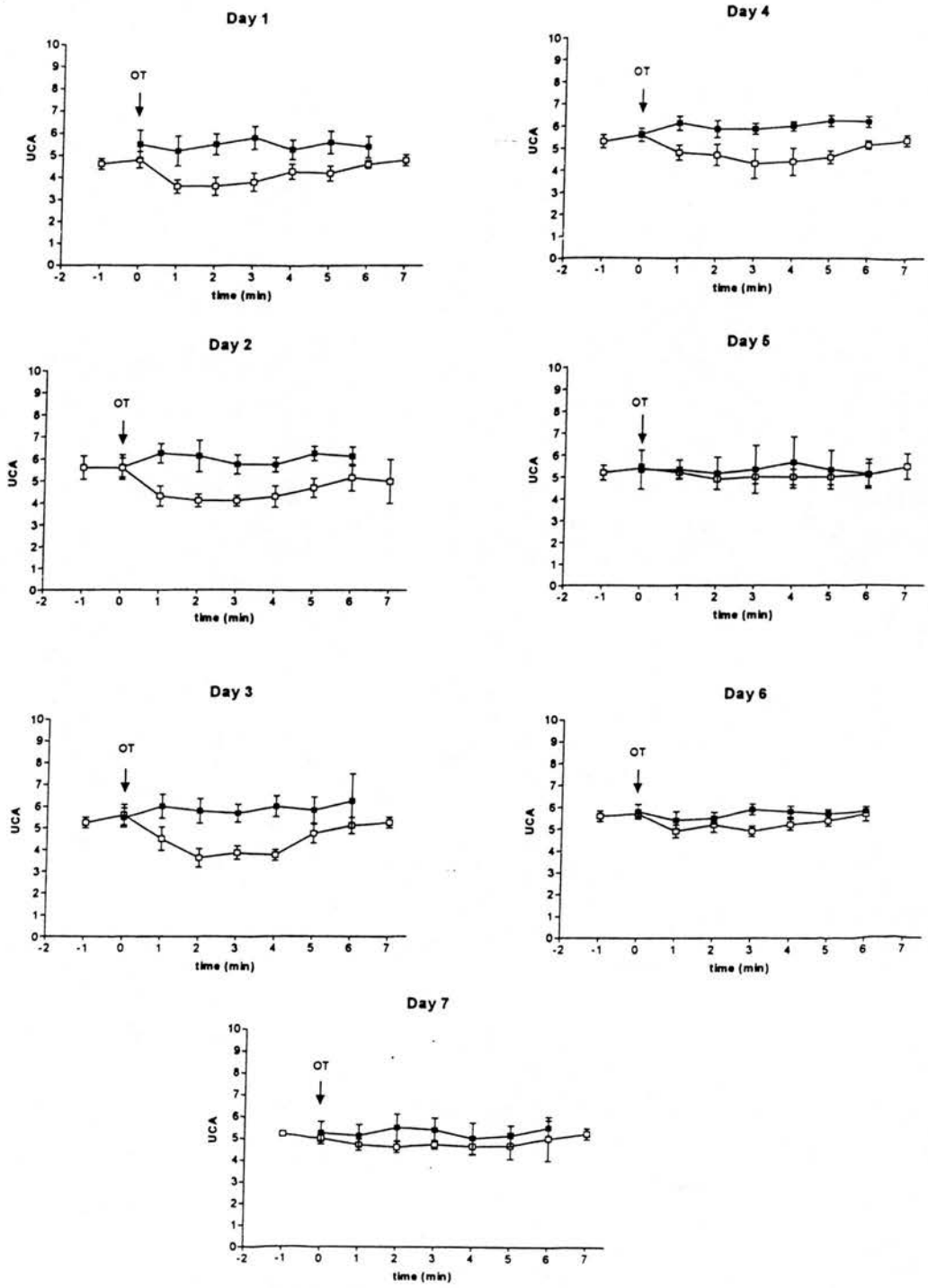

Figure 8.1. Uterine activity from D1 to D7 after ovulation for the control (■) (n=5) and the oxytocin treatment (□) (n=5) cycle.

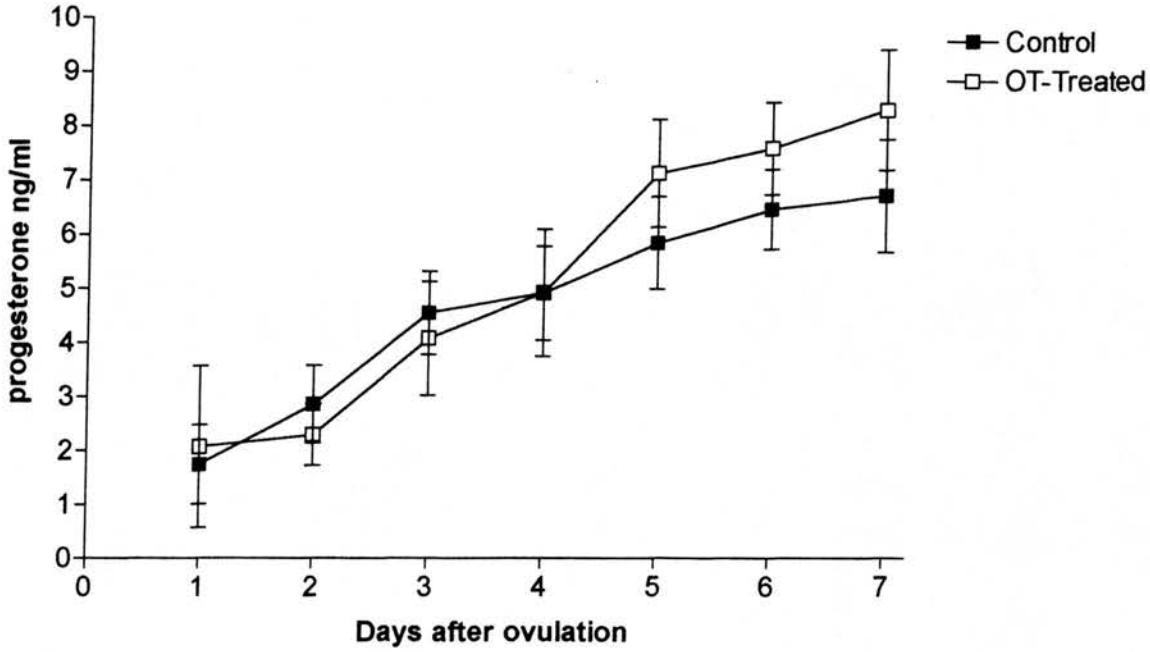

Figure 8.2. Mean daily progesterone levels from D1 to D7 after ovulation for the control (■) and the oxytocin treatment (□) cycle. Ovulation is designated as day 0.

However, on day 3, UCA scores did not significantly decrease before the second minute after OT administration, indicating a delayed uterine response. For days 1, 2 and 3, uterine motility scores returned to baseline levels by 5 minutes after OT administration. The administration of OT did not have any significant effect on UCA scores after day 3 postovulation.

Discussion

In the mare progesterone concentrations rise within 12 to 24 hours after ovulation (Plotka *et al.*, 1975; Townson *et al.*, 1989) reaching high dioestrous values by day 5 to 7 after ovulation, until their rapid decline at luteolysis between days 14 and 16

(Stabenfeldt *et al.*, 1972). In this study the patterns of circulating progesterone concentrations observed during the period of luteal development were similar to previous reports (Plotka *et al.*, 1975; Pipkin *et al.*, 1987; Townson *et al.*, 1989). In the present study administration of therapeutic doses of OT in the first seven days postovulation failed to affect concentrations of circulating progesterone. These results agree with the findings of Neely and coworkers (1979a) where daily administration of 150 IU oxytocin later in the oestrous cycle, from days 4-8 postovulation, failed to influence the function of the CL . Also Goff and coworkers (1987) reported that the administration of a smaller OT dose (20iu/500kg) on alternate days from day 9 did not affect the length of the oestrous cycle. In the present study the mares were short-cycled and so oestrous cycle length was not recorded. However, based on the similarity of the progesterone profiles between the two groups of mares, it is unlikely that treatment would have affected cycle length. Presumably the failure to affect CL function in this study was due to the low capacity of OT to release endometrial PGF2α in early dioestrus (Goff *et al.*, 1987), combined with the insensitivity of the early CL to the luteolytic action of PGF2α (Douglas and Ginther, 1972). It would also appear from the results of this study that OT does not have a direct effect on the CL. The results of this chapter are particularly interesting because of the widespread use of OT as a postbreeding therapy and because of the potentially greater sensitivity of the developing CL to interference with growth and development than the fully developed CL. However, in this study the possible effect of multiple therapeutic daily OT injections on CL function was not investigated.

In the present study transrectal ultrasonography was the method selected to monitor UCA after the administration of OT in the mare. The study of UCA has been greatly aided by the use of ultrasonographic equipment which enables the direct visualization of subendometrial myometrial contractions. Uterine motility is seen as an endometrial "wave motion", bearing a strong similarity to gut motility. The scale used to score uterine motility in the present study was based on previous studies in women (Abramowicz, 1990; Lyons *et al.*, 1991; Salamanca and Beltran, 1995; Kunz *et al.*,

1996; Leyendecker *et al.*, 1996) and mares (Cross and Ginther, 1987; Cross and Ginther, 1988; Griffin and Ginther, 1990; Gastal *et al.*, 1998). In women it is possible to study the direction of peristaltic waves however this was not possible in mares because of the lack of organized contractions in early dioestrus. In this study the technique of ultrasonography appeared not to affect UCA. However, although rectal palpation did not significantly alter electromyographic recordings (Taverne *et al.*, 1979b), ultrasonography initially increases myometrial electrical activity in the mare (M.Troedsson personal communication). Electromyography is likely to be more sensitive in detecting small changes in uterine activity that cannot be visualised when observing UCA by ultrasonography.

In the present study baseline UCA did not significantly change from day 1 to day 7 after ovulation. These findings are further supported by Sharp et al. (1997) who reported no difference in the concentration and affinity of uterine OT receptors in nonbred mares from oestrus to day 8 after ovulation. In another ultrasonographic study in nonbred mares (Griffin and Ginther, 1990), UCA decreased between days 0 and 1 and increased between days 2 and 4. In this study UCA was followed only after ovulation and no statistically significant difference between any of the days was found. It could be that the differences between the two studies are due to the different scoring system used and not to actual differences in UCA.

In previous studies, the effect of OT on uterine smooth muscle activity has been demonstrated utilizing different methods and different doses of OT (Goddard and Allen, 1985; LeBlanc *et al.*, 1994b; Troedsson *et al.*, 1995e; Pycock *et al.*, 1997). Intrauterine pressure and electromyographic studies have shown that the administration of OT causes a response within 1 minute (Goddard and Allen, 1985; Jones *et al.*, 1991). This response subsides between 10 minutes (Goddard and Allen, 1985) and 1 hour (Troedsson *et al.*, 1995e) at all stages of the cycle (Goddard and Allen, 1985; Ko *et al.*, 1989). In another study, different patterns of myometrial electrical activity were recognized in oestrus and dioestrus (Troedsson *et al.*, 1993a) and administration of OT resulted in a less

pronounced response in dioestrus than in oestrus (Jones *et al.*, 1991). The administration of OT also caused the expulsion through the cervix of more than 90% of infused radiocolloid in late oestrous and early dioestrous (day 2 postovulation) mares (LeBlanc *et al.*, 1994b). In the present study, the administration of OT did not cause the dramatic uterine contraction that was observed by ultrasonography in chapter 7 where OT was administered to oestrous mares. In that study, a greater degree of uterine response in the form of uterine spasm was detected within 1 minute of OT administration and UCA returned to baseline levels within 6 minutes. The present study suggests that the diminished effect of OT on UCA after day 3 results from changes in OT receptors at this stage of the cycle. Previous work suggests that uterine OT receptors tend to increase during dioestrous and peak at luteolysis (Goff *et al.*, 1987; Sharp *et al.*, 1997). However, detailed studies have not been performed in the early postovulatory period. The findings of the present study are in agreement with other reports where OT administration provoked uterine contractions in the early postovulatory period (Ko *et al.*, 1989; LeBlanc *et al.*, 1994b). However this is the first study to the knowledge of the author that follows the daily effect of OT administration on UCA during the early postovulatory period.

The diminished effect that OT has on UCA after day 3 of dioestrus, according to the findings of the present study, suggests that treatment of mares with PMIE with OT after day 3 may be less effective than early treatment. These results confirm the recommendations made by other workers (LeBlanc, 1997b; Knutti *et al.*, 1997) that early treatment (within the first 12 hours from insemination) of these mares is essential. The effect of higher OT doses on UCA after day 3 should be investigated in combination with studies on the effect of OT on uterine clearance and cervical drainage.

It was concluded that daily OT administration at a commonly used therapeutic dose did not affect circulating progesterone levels. Also, OT administration significantly affected uterine motility up to day 3 after ovulation, after which there was no difference between

the control and OT treated cycles. Further investigations into the effect of higher OT doses on UCA after day 3 postovulation are needed.

Chapter 9

General Discussion

Uterine contractile activity represents the final stage in a series of complicated processes at a cellular level. Uterine contractile activity is an indispensable part in a complex mechanism of uterine defences, which is responsible for keeping the homoeostatic balance of the uterine environment. In this discussion, UCA is going to be considered from two different aspects, in the light of the results presented in this thesis. Firstly, UCA will be discussed as the result of a multidimensional mechanism involving the physiology of muscle contractility and especially its control, and secondly, the role of UCA as a factor of stability in the uterine environment.

The control of UCA is based on three separate, but interacting, mechanisms. Myogenic control of UCA is expressed at a cellular level and has to do with the physiology of uterine contraction at a cellular level. Neurogenic control involves the control of the adrenergic, cholinergic and peptidergic nervous system on UCA. Hormonal control of UCA, which appears to be the most effective, involves the steroid hormones, oestrogen and progesterone, and the ecbolic hormones, OT and $PGF_{2\alpha}$. These three mechanisms appear to control UCA and interact in an ascending order which will be discussed below.

The basic physiology of uterine smooth muscle contraction has been extensively explored and described (Csapo, 1950; Csapo and Gergely, 1950; Csapo, 1956a; Csapo, 1962; Marshall, 1970; Garfield *et al.*, 1985), showing little, if no, species differences. The ability of myometrial cells to contract depends upon the distribution of ions across their plasma membranes. The ionic distribution in uterine smooth muscle is such that sodium and calcium ions are higher outside the cell than inside (Kao 1989). Electrical and chemical changes occurring within the muscle cell cause voltage and time dependent changes in membrane ionic permeability. Changes in the membrane permeability allow calcium to enter the muscle cell, interact with the myofilaments and cause uterine contraction (Garfield *et al.*, 1985). These changes are initiated by pacemaker cells, which are thought to be responsible for initiation of spontaneous electrical activity in uterine smooth muscle. Although it was initially thought that non-

pacemaker regions would be excited only when the firing threshold was reached in the pacemaker cell area, it was shown that any non-pacemaker region can become pacemaker by the application of OT, acetylcholine or prostaglandins (Lodge and Sproat, 1981). The propagation of electrical activity between uterine smooth muscle cells is facilitated by gap junctions, intercellular channels that link cells to their neighbours by allowing the passage of inorganic ions and small molecules (Ichikawa and Bortoff, 1970; Decker, 1976; Fry *et al.*, 1977; Garfield *et al.*, 1980b; Garfield *et al.*, 1988). Gap junctions are also responsible for the coordination of uterine contraction, especially around parturition (Garfield *et al.*, 1977; Saito *et al.*, 1985).

The uterus is innervated by adrenergic and cholinergic nerves entering from the tubal end. However, the uterine innervation has not been considered as an important regulator of UCA since there is no recognized conduction pathway in the myometrium, comparable to the Purkinje fibre system in the heart (Garfield *et al.*, 1994). Uterine contractile activity has also been shown to be modulated by different peptides. Peptides appear to play a role in the control of UCA by affecting it directly or indirectly. Vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY) and nitric oxide (NO) have been reported to inhibit myometrial contractility (Ottensen *et al.*, 1981; Diamond, 1983; Ottensen *et al.*, 1983; Stjernquist *et al.*, 1983). Substance P (SP) increases the smooth muscle tension and calcitonin gene-related peptide (CGRP) diminishes spontaneous contractions (Samuelson *et al.*, 1985), while galanin and gastrin-releasing peptide (GRP) cause uterine contraction (Stjernquist *et al.*, 1986; Stjernquist *et al.*, 1988). However, there is strong evidence suggesting that ovarian steroids have a detrimental effect on autonomous uterine innervation (Falck *et al.*, 1974; Falck *et al.*, 1975), especially during pregnancy (Thorbert, 1978).

Hormonal control is the third and last mechanism controlling UCA and consequently uterine function. As mentioned above, ovarian hormones can modify the neurogenic and also affect the myogenic control of UCA. Levels of ovarian steroids change in response to administered hormones, or physiologically, with oestrous and seasonal

cyclicity, pregnancy and parturition. Despite the great differences in seasonal cyclicity amongst species, the effect of the ovarian hormones on UCA are characteristic. In the next paragraphs the effects of ovarian steroids on parameters of UCA will be discussed.

Oestrogen has been shown to control the two key elements to UCA: intracellular calcium concentrations (Bozler, 1941; Marshall and Csapo, 1961; Csapo 1981) and gap junction formation (Burghardt and Matheson, 1982; Wathes and Porter, 1982; MacKenzie *et al.*, 1983; Burghardt *et al.*, 1987). Also recent reports have shown that oestrogen affects uterine blood flow (Penny *et al.*, 1981; Kostrzewska *et al.*, 1988). Oestrogen and diethylstiibestrol treatments increased influx of calcium (Batra 1987), and the size and number of gap junctions in the myometrium (Bergman, 1968; Dahl and Berger, 1978; Merk *et al.*, 1980). However in the mare, the presence of gap junctions in the endometrium did not seem to be affected by the day of the oestrous cycle (Brady *et al.*, 1995). This has been attributed to the action of several protein kinases, that are activated in response to different physiological stimuli (Saez *et al.*, 1990).

Progesterone, by contrast, has a strong inhibitory effect on spontaneous myometrial activity (Kostrzewska *et al.*, 1993) and on uterine blood flow (Resnik, 1977; Batra, 1985). It has been suggested that the inhibitory effect of progesterone is exerted by its metabolites which appear to interact with the receptor of gamma-aminobutyric acid (GABA) (Erdo, 1984; Majewska and Vaupel, 1991). Progesterone supresses the formation of gap junctions (Ichikawa and Bortoff, 1970; Decker, 1976; Garfield *et al.*, 1980a; Garfield *et al.*, 1980b) however it seems to have no effect on calcium uptake (Batra 1994). This suggests that UCA in a progesterone-dominated uterus is subject to other regulatory mechanisms that still remain unknown.

All these remarks have been concerned with the ultrastructural mechanisms responsible for the control UCA. As was stated earlier, UCA plays an important role as a uterine defence mechanism in conjunction with the cellular and antibody-mediated defence mechanisms.

Cellular and antibody-mediated defence mechanisms are responsible for the killing and ingestion of bacteria in the uterus by neutrophils and UCA is responsible for the mechanical clearance of uterine fluid and cellular debris through cervical and lymphatic drainage (Guyton 1991). The synchronized and complementary function of these three mechanisms ensures an inflammation-free uterine environment that will guarantee the viability of the embryo at the time of its descent into the uterus.

This homeostatic function of UCA is of particular interest and importance in the mare, where overall low fertility rates (Rossdale *et al.*, 1980) are further reduced by subfertility and early embryonic death, both usually due to endometritis (Knudsen, 1964b; Varadin, 1975; McKinnon *et al.*, 1994; Mattoras *et al.*, 1995). The endometritis model is based on the assumption that the three defence mechanisms contribute equally to the maintenance of a healthy uterine environment. The role of UCA in gamete transport after breeding and the physical removal of cellular debris and intrauterine fluid that accumulate after breeding.

The first objective of the present thesis was to determine the importance of UCA in the clearance of experimentally-induced uterine infection in genitally normal mares and mares with reduced UCA. The causal relationship between impaired UCA, delayed uterine clearance and PMIE is well known in the susceptible mare (Evans *et al.*, 1987; Troedsson and Liu, 1991; Troedsson *et al.*, 1993b; LeBlanc *et al.*, 1994a; LeBlanc *et al.*, 1995c; see Chapter 7). Several investigators in the past have reported contradictory results concerning the role of cellular and antibody-mediated defence mechanisms in mares resistant and susceptible to PMIE (see Chapter 1). However there is general agreement that mares susceptible to PMIE fail to clear intrauterine fluid accumulations and this has been attributed to impaired UCA.

The results presented in this thesis add further to the theory of impaired UCA in mares susceptible to PMIE. The importance of UCA for the physical elimination of infection

was demonstrated by the use of clenbuterol, a β_2 sympathomimetic smooth muscle relaxant. The interference with the neurogenic control of UCA resulted in decreased UCA, imitating the impaired UCA of susceptible mares and demonstrated the accumulation of intrauterine fluid. Additionally, the mares used were classed as genitally normal prior to the experiment and had cleared the introduced infection successfully in a previous cycle. Furthermore, bacteriology results showed that bacteria were eliminated in 3 of 5 mares suggesting functional antibacterial mechanisms. These results clearly demonstrate the importance of UCA but also suggest that its role might be more vital in the elimination of uterine infection than the other defence mechanisms. However, until a model can be applied where the course of uterine infection can be followed when cellular and humoral defences are blocked, this remains uncertain.

Assuming that mares resistant and suceptible to PMIE have the same myogenic and neurogenic controls *ab constructio*, emphasis was given to the hormonal control of UCA. The investigation of ecbolic hormone profiles around different reproductive events and uterine manipulations gave insight to several aspects of the mare's reproductive physiology as well as to the secretion patterns of these hormones.

Oxytocin has long been known to be released in response to vaginocervical stimulation. In the mare, it was firstly reported in a treaty by Xenophon (4^{th} century BC), where he described that air blown into the mare's vagina would elicit milk let-down. In women this was described as the Ferguson reflex (Ferguson, 1941). This knowledge has been broadly used in several species to monitor OT release and interactions with other systems. In this thesis it was shown that not only mechanical stimuli such as mating and manipulation of the genital tract caused OT release. On several occasions visual stimuli such as the sight of the stallion initiated OT release. Also on numerous occasions OT peaks coincided with vocalisations of the mare, urination, sight of food or stress, however these results were not statistically analysed. Similar observations have been reported in the ewe (Lehrer *et al.*, 1978) and the mare (Taverne *et al.*, 1979b). This

suggests that OT release is based not only in an internal circadian pulsatile rhythm but also in an interactive manner dependent on the external environmental stimuli.

Oxytocin release is a pulsatile phenomenon and different modes of "spurt" release have been described in ruminants and the mare (Burns *et al.*, 1981; Tetzke *et al.*, 1987; Stevenson *et al.*, 1991; Sharp *et al.*, 1997). In ruminants OT is released from the ovary and initiates the lysis of the corpus luteum by triggering prostaglandin release (Wathes 1989). However, this is not the case in the mare, where OT is released centrally only from the posterior pituitary (Vanderwall *et al.*, 1998). In the present thesis no specific patterns of OT release were established. The experiments reported in this thesis were designed to investigate the effect of different stimuli on OT release and not to monitor regular OT release. There seems to be a gap in the current literature on this subject. Reports on OT release patterns in the mare are contradictory and therefore misleading either because the methods used to extract OT from blood plasma had low reproducibility (Burns *et al.*, 1981; Tetzke *et al.*, 1987) or because of infrequent blood sampling regimes (Stevenson *et al.*, 1991). A detailed study of normal OT profiles throughout the oestrous cycle and especially around ovulation is of great importance for a deeper understanding of OT release patterns in the mare.

In the present thesis it was demonstrated that a broad variety of olfactory, acoustic, visual, tactile and psychogenic stimuli involved in events such as teasing, can affect OT release. Also it was demonstrated that although a stimulus elicited OT release in two different stages of the oestrous cycle it initiated different types of behaviour. Teasing in oestrus increased sexual receptivity and initiated sexual behaviour while teasing in dioestrus had diametrically opposite results; the mare strongly rejected the stallion. This observation raises two questions. Firstly, what is the role of OT in the initiation of different behaviours in the mare; and secondly, which are the factors that will promote the manifestation of one type of behaviour over another?

Recent reports on different species support the involvement of OT in memory, compulsive eating disorders and different types of behaviour (Witt and Insel, 1990; Argiolas and Gessa, 1991; Insel, 1992; de Wied *et al.*, 1993; Leckman *et al.*, 1994; Landgraf, 1995), such as sexual and maternal (Argiolas *et al.*, 1988; Witt and Insel, 1991; Carter, 1992; Caldwell *et al.*, 1994a; Caldwell *et al.*, 1994b; Nishimori *et al.*, 1996; Insel *et al.*, 1997). Although the release of OT around teasing and breeding in the mare may be important peripherally, by enhancing gamete transport and evacuation of uterine contents after mating, its role centrally, in initiating sexual behaviour, remains to be investigated. It also appears that the concentration of ovarian steroids can significantly affect the type of promoted behaviour. In the presented work, increased oestrogen levels promoted sexual receptivity while rising progesterone levels at day 7 of dioestrus promoted sexual rejection. It could be that the steroid environment affects behavioral responses to somatosensory stimuli (Caldwell *et al.*, 1996). However further investigation is required into this subject.

Artificial insemination was another stimulus that was applied in order to investigate possible differences in OT profiles between resistant and susceptible mares. No differences were found between the OT profiles of the two groups of mares. What was striking though, was the complete lack of $PGF_{2\alpha}$ release in response to AI in mares susceptible to PMIE. Furthermore, the administration of exogenous OT failed to elicit prostaglandin release, in sharp contrast with mares resistant to PMIE. This particular finding is of great importance since it could be the first step towards a diagnostic step in the early prediction of susceptibility to uterine infection in the mare.

Prostaglandins are released from the equine endometrium as a result of inflammation, trauma or manipulation. Uterine inflammation, an inevitable sequel of breeding, activates the humoral and cellular defence mechanisms that "attack" and neutralize the intruder. Inflammatory mediators released during this process trigger the arachidonic acid cascade that results in the release of prostaglandin which in turn enhances UCA and uterine clearance. Prostaglandins have also been shown to stimulate the formation of

gap junctions (MacKenzie and Garfield, 1985). Furthermore in the mare, blocking prostaglandin release with phenylbutazone has been shown to significantly delay uterine clearance of radiocolloid (Cadario *et al.*, 1995).

Pulses of OT activate $PGF2\alpha$ synthesis mediated via the uterine OT receptor. This has been described at different stages of the oestrous cycle in the mare and it was shown that in oestrus the $PGF2\alpha$ response is lowest (Goff *et al.*, 1987). Endogenous OT pulses initiate $PGF2\alpha$ release at around day 14 to 16 after ovulation leading to luteolysis. A temporal relationship between OT and $PGF2\alpha$ has been demonstrated in several species, however reports in the mare remain contradictory. Prostaglandin has been reported to precede the OT pulse, however recent reports contradict the view by showing $PGF2\alpha$ pulses to coincide or follow OT pulses (Madill *et al.*, 1998). In chapters 4 and 5, where ecbolic hormone release is described, although OT release was observed on most occasions, the incidence of $PGF2\alpha$ release was much lower in resistant mares whereas susceptible mares consistently failed to release $PGF2\alpha$. It could be that endometrial functional and structural disturbances in susceptible mares (Kenney, 1978) are responsible for the malfunction of uterine OT receptors. Another possible explanation could be the depletion of arachidonic acid depots, that are responsible for uterine prostaglandin synthesis. This could be due to the presence of PGH synthase (Poyser, 1995), an endogenous inhibitor of prostaglandin synthesis, that has been found also in the mare (Watson, 1991). This however awaits further investigation.

The combined effectiveness of the uterine defence mechanisms can be easily assessed from the results of endometrial cytology and bacteriology examination. The introduction of semen in the uterus at the time of natural service causes an inflammation that mares susceptible to PMIE fail to resolve within 48 hours. In order to reduce the challenge for the susceptible mares, AI with extended semen and antibiotics has been recommended and is broadly used (Kenney *et al.*, 1975). However in this thesis it was shown that AI in susceptible mares resulted in a severe inflammatory response and persistent endometritis. It has been suggested that AI in mares susceptible to PMIE

should be followed 4-12 hours later by uterine flush and OT treatment which has been shown to increase fertility rates (LeBlanc and Asbury, 1994; Knutti *et al.*, 1997).

One of the aims of this project was to look closely at the effect that different current therapeutic and diagnostic attitudes have on UCA and in this way add an element of practicality and applicability to the presented work. Oxytocin treatment of PMIE is a widely used therapy, aimed at enhancing UCA and increasing uterine clearance. Sometimes the repeated use of OT injections in parallel with uterine flushing and antibiotic treatments (Pycock and Newcombe, 1996a) is indicated in order to achieve satisfactory results. In this thesis, ultrasonography was used to monitor and quantify the effect of OT on UCA. In a preliminary study, it was ensured that ultrasound scanning did not in itself cause ecbolic hormone release and alteration to UCA, validating ultrasonography as an applicable method for the quantitation of UCA. This is of great importance since ultrasonographic equipment is a common facility in most equine practices and the development of a susceptiblity prediction test based on ultrasonography would greatly improve the provided services.

The differences in baseline UCA scores presented between resistant and susceptible mares give a different perspective of UCA in the mare. In an oestrogen-dominated uterus resistant mares had significantly higher UCA scores than susceptible mares. In an electromyographic study (Troedsson *et al.*, 1993b) no differences were found in the intensity, frequency and duration of uterine contractions between resistant and susceptible mares. However, in the same study asynchronous contractions were observed more often in susceptible mares than in resistant. So, could it be that impaired UCA in susceptible mares does not depend on the inability of the myometrium to contract but it rather depends on its inablility to contract in a synchronized manner? It has been shown in the mare and other species that the inner circular and the outer longitudinal uterine muscle layers respond differently to separate stimuli. Elevated oestrogen levels at the time of oestrus increase myometrial cellular communication, as discussed previously, by increasing number and size of gap junctions. Uterine

contractile activity in resistant oestrous mares, is visually perceived as coordinated wave motion, that involves a relaxation-contraction pattern. By contrast, UCA in susceptible mares appears to be less coordinated and did not display the typical pattern of contraction. It could be that this is due to the inability of the two uterine muscle layers to coordinate their contraction patterns in such a way as to create a peristaltic uterine contraction that will lead to the successful explulsion of intrauterine fluid.

However, both resistant and susceptible oestrous mares respond to OT administration with a strong uterine spasm. This shows that exogenous OT, in concentrations that by far exceed physiological levels, causes a uniform reaction in both resistant and susceptible oestrous myometria. Interestingly, as OT concentrations decrease in the peripheral circulation after exogenous OT administration, UCA returns to its baseline levels faster in resistant than in susceptible oestrous mares. It could be that the ability of the uterus to recover faster after uterine spasm caused by a high OT dose, is a sign of well coordinated activity of the two uterine muscle layers.

In early dioestrus, there were no differences in UCA scores between resistant and susceptible mares and overall UCA scores were significantly lower than in oestrous resistant mares. Also daily OT administration at a commonly used therapeutic dose did not affect circulating progesterone levels. Bearing in mind the effect that progesterone has on UCA, lower UCA scores in early dioestrus are easily explained by the rising progesterone concentrations following ovulation. The research presented in this thesis into the myometrial response to OT administration in the early postovulatory period has given results that are valuable not only from a scientific but also from a practical point of view. It was shown that the use of OT after day 3 postovulation does not affect UCA. It was also shown that the repeated administration of OT in oestrous mares had a markedly lower effect on UCA scores after its first application, suggesting uterine refractoriness. This information could be used in the planning of breeding and treating schemes for susceptible mares in order to save valuable treatment time and expenses for both the horse breeder and the equine practitioner.

In conclusion most of the outlined objectives of this thesis were successfully answered. However those answers have raised new questions about the reproductive biology of the mare and have also challenged standard clinical treatments. Clenbuterol was used successfully to decrease UCA and compromise the uterine defence mechanisms of genitally normal mares. In parallel, clenbuterol is being used in greater doses and for longer periods of time in COPD mares. Could it be that treatment with clenbuterol compromises the fertility of these mares? In the present work, the different ways in which OT administration affects UCA were presented according to the stage of cycle and the mare's classification as resistant or susceptible to PMIE. Further research is needed however to correlate these findings with actual uterine clearance. In this thesis OT profiles of resistant and susceptible mares were demonstrated around different reproductive events. The work presented in this thesis and the contradictory findings in the literature on OT profiles in the mare have indicated the need for a detailed study of daily OT profiles, especially around ovulation. Also the role of OT in the behaviour of the mare is a field that deserves attention.

The differences in prostaglandin release and UCA scores betweeen resistant and susceptible mares definitely bring us a step closer towards understanding the pathophysiological mechanisms underlying endometritis and could serve in the future as a predictive test for the detection of endometritis.

A computerized approach to UCA could also help in the understanding of contraction patterns of the two separate uterine muscle layers. Sequences of images obtained from an ultrasound scanning and recorded on video tape can be digitised and analysed utilizing inter-frame analysis measuring the distribution of displacement of images from one frame to the next. Sequences of vector maps can be created and then be further processed in a variety of ways in order to detect, classify and quantify various types of contraction and peristaltic motion in mares resistant and susceptible to PMIE.

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Publications

Publications arising from this thesis:

Abstracts

1. **Nikolakopoulos, E. and Watson, E.D.** (1997) Plasma oxytocin levels around natural service and teasing in the mare. *Journal of Reproduction and Fertility Abstract Series* **19**, Abstract 77

2. **Nikolakopoulos, E. and Watson, E.D.** (1997) Uterine motility in mares resistant and susceptible to endometritis *Journal of Reproduction and Fertility Abstract Series* **20**, Abstract14

3. **Nikolakopoulos, E. and Watson, E.D.** (1997) Uterine inflammatory response to breeding and ecbolic hormone concentration in mares resistant and susceptible to endometritis *Pferdeheilkunde* p. 532

Refereed articles

1. **Nikolakopoulos, E. and Watson, E.D.** (1997) Does artificial insemination with chilled extended semen reduce the antigenic challenge to the mare's uterus compared with natural service? *Theriogenology* **45**:583-590.

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3. **Nikolakopoulos, E., Kindahl, H. and Watson, E.D.** (1998) Oxytocin and $PGF_{2\alpha}$ release in mares resistant and susceptible to persistent mating-induced endometritis. *Journal of Reproduction and Fertility Supplement (in press).*